Subunit Composition of Rabbit Muscle Phosphoglucomutase†

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ABSTRACT: Rabbit muscle phosphoglucomutase, hitherto regarded as a single polypeptide of molecular weight about 62,000 g/mole, is shown to consist of two subunits of about equal size. Guanidine hydrochloride will not dissociate these subunits, unless the sulfhydryl groups are carboxymethylated, in which case partial dissociation occurs. Sodium dodecyl sulfate at 0.10% converts the protein completely into half-molecules, whose size has been measured by the sodium dodecyl sulfate–polyacrylamide gel technique and also by high-speed equilibrium centrifugation. The dansylation procedure indicates that phosphoglucomutase contains two amino-terminal amino acids, valine and lysine. One mole of valine can be found by the carbamylation method, while lysine is undetectable. The two subunits thus seem to have differences in their primary structures, a property expected from the peptide mapping studies of previous workers. The phospho form of phosphoglucomutase, that generally employed in studies of this enzyme, is considerably more resistant to denaturation than the dephospho form, and it was the use of dephosphoenzyme which allowed demonstration of amino-terminal residues and of subunits in this enzyme.

Phosphoglucomutase from rabbit muscle has generally been regarded as a monomeric enzyme of molecular weight about 62,000 g per mole (Filmer and Koshland, 1963; Yankeelov *et al.*, 1964; Joshi *et al.*, 1967; Harshman and Six, 1969). Carboxymethylation of the enzyme's sulfhydryl groups after disulfide reduction, followed by denaturation in 5 M guanidine hydrochloride, fails to lower this molecular weight (Harshman and Six, 1969), and peptide mapping of tryptic digests of the enzyme indicates that if subunits exist, they must be chemically different (Joshi *et al.*, 1967; Joshi and Handler, 1964, 1969).

These conclusions are reinforced by similar observations made, in part, on the homologous enzymes from human muscle (Joshi and Handler, 1969), flounder and shark muscle (Hashimoto and Handler, 1966), and the phylogenetically remote *Escherichia coli* cells (Joshi and Handler, 1964). Isoenzymes of phosphoglucomutase have been demonstrated in rabbit muscle (Yankeelov *et al.*, 1964; Joshi *et al.*, 1967; Harshman and Six, 1969) as well as in several other mammalian sources (references in Harshman and Six, 1969; Joshi and Handler, 1969), but it is clear that one principal enzyme form comprises about 80% of the total, while three others are present in about equal amounts (Harshman and Six, 1969). The basis for the differences between these isoenzymes is unknown.

Phosphoglucomutase from many sources can exist in either a phosphorylated or a dephosphorylated form. The amino acid sequence around the phosphoseryl residue involved has been established for the rabbit muscle enzyme (Hooper *et al.*, 1968; Milstein and Milstein, 1968). The processes of attachment and removal of this phosphate group appear to be integral parts of the catalytic mechanism (Najjar and Pullman, 1954; Najjar, 1962; Ray and Roscelli, 1964; Peck *et al.*, 1968). The phosphoenzyme and dephosphoenzyme display certain differences which suggest that they represent different conformational states. The dephosphoenzyme is considerably more sensitive to heat (Najjar, 1962; Alpers and Lam 1969), and some of its sulfhydryl groups react more readily with *p*-hydroxymercuribenzoate than do the corresponding groups in the phosphoenzyme (Bocchini *et al.*, 1967). The two enzyme forms also differ considerably in optical activity, even when this difference is measured at the insensitive wavelength of 590 nm (Robinson and Najjar, 1961). Bocchini *et al.* (1967) have reasonably argued that the experiments of Yankeelov and Koshland (1965), interpreted in terms of the induced-fit hypothesis (Yankeelov and Koshland, 1965), actually provide further evidence that the phospho- and dephosphoenzyme exist in significantly different conformations.

Our discovery that rabbit muscle phosphoglucomutase is subject to allosteric inhibition by several nucleotides (H. W. Duckworth and B. D. Sanwal, in preparation) led us to reinvestigate the subunit structure of this enzyme. The phosphoenzyme is the form obtained by the usual purification procedures, probably because of the heat treatments employed (Najjar, 1955, 1962), and this form is remarkably resistant to denaturation. Apart from its stability to heat already mentioned, one may note the failure of previous workers to detect any amino-terminal (Joshi and Handler, 1964; Milstein and Sanger, 1961; Harshman *et al.*, 1963) or carboxyl-terminal residues in the phosphoenzyme (Harshman *et al.*, 1963). These facts prompted us to choose the dephospho form of rabbit muscle phosphoglucomutase for intensive study, and unless otherwise stated the results presented in this paper were obtained exclusively with this form.

Experimental Section

Materials. Essentially all of the studies reported in this paper were performed on one lot of crystalline rabbit muscle phosphoglucomutase (Sigma lot 119B-4740) which was chromatographed on CM-cellulose according to Yankeelov *et al.* (1964) to separate the phospho and dephospho forms. These were then stored in 0.04 M sodium acetate (pH 5.0) which was 2.75 M in ammonium sulfate. We have confirmed the report that commercial enzyme contains equal amounts of the two forms (Alpers and Lam, 1969). Specific activities of the two fractions in the routine assay (see below) were initially 250–

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350 units/mg of enzyme, and had declined to about 50 units/mg by the end of the study. The values initially found correspond to specific activities of 475–665 U/mg at 30°, the temperature often used in published work, as is explained below in the details of our routine assay. Thus our phosphoglucomutase is of comparable specific activity to the best preparations described by others (see Ray and Roscelli, 1964). Detailed evidence for the homogeneity of this preparation is given under Results. Amino acid analyses of the usual acid hydrolysates of the preparation were in good agreement with the more recent literature values (Yankeelov *et al.*, 1964; Joshi *et al.*, 1967; Harshman and Six, 1969).

Guanidine hydrochloride was Mann (Ultra Pure grade, lot V-3379). Sodium dodecyl sulfate labeled with ^{35}S was obtained from Amersham-Searle. All other chemicals were reagent grade.

Molecular Weight Determinations. Electrophoresis in the dodecyl sulfate gels, and the preparation of the protein samples for this procedure, were performed according to Weber and Osborn (1969), with several modifications. Horse heart cytochrome *c* was used as a visible marker instead of tracking dye, and protein positions were determined by measuring optical density scans of the Coomassie Blue stained gels. No stacking gel was used. In our work, it was necessary to add 0.1% 2-mercaptoethanol to the running buffer, and prerun the gels for 2 hr at 8 mA/tube before loading the proteins, to remove traces of persulfate catalyst from the gels, as is explained under Results.

Ultracentrifugations were done with a Beckman Model E analytical ultracentrifuge equipped with interference optics and a specially designed speed control. All experiments were at 20°, at a rotor speed of 29,851 rpm. High-speed equilibrium data (Yphantis, 1964) were measured from the usual photographs of Rayleigh interference fringes, and were processed using the computer program of Roark and Yphantis (1968) to obtain weight-average molecular weights as a function of protein concentration. Samples were prepared by dialyzing small samples of protein, at about 0.5 mg/ml, against large volumes of buffer, guanidine hydrochloride, or the dodecyl sulfate at the required concentrations. In the case of the dodecyl sulfate, the protein samples were found to be concentrated to a variable degree (5- to 10-fold) during dialysis, and these were diluted with dialysate to a suitable level for interference measurements after measuring the protein concentration by the method of Lowry *et al.* (1951).

Molecular weights of proteins in the dodecyl sulfate must be corrected for the binding of sodium dodecyl sulfate, which is substantial. The binding affects the determination in two ways. First, the buoyancy of the protein–sodium dodecyl sulfate complex is greater than that of free protein, since the partial specific volume of the dodecyl sulfate is considerably greater than that of normal proteins. Second, the molecular weight of the complex is much larger than that of the protein, since so much sodium dodecyl sulfate is bound. The extent of binding of the dodecyl sulfate to the enzyme was estimated by equilibrium dialysis using ^{35}S-labeled sodium dodecyl sulfate at a free sodium dodecyl sulfate concentration of 0.10%, in 0.1% 2-mercaptoethanol and 0.01 M sodium phosphate, pH 7.0 (μ = 0.02). Molecular weight data obtained in this medium were corrected for this binding according to Carusi and Sinsheimer (1963).

The partial specific volume, $\bar{V}$, of the enzyme in buffer or guanidine hydrochloride solutions was taken to be 0.7383 ml/g, the value obtained from the amino acid composition by assuming simple additivity of the specific volumes of the amino acid residues (Cohn and Edsall, 1943). The value $\bar{V}$ for the enzyme in the dodecyl sulfate was calculated according to Carusi and Sinsheimer (1963), assuming additivity of the specific volume of the enzyme (0.7383 ml/g) and the dodecyl sulfate (0.876 ml/g). Carusi and Sinsheimer (1963) cite published evidence which justifies this additivity assumption.

Reduction and Carboxymethylation of the Enzyme. Protein at about 10 mg/ml was incubated 4 hr at 60° in 6 M guanidine hydrochloride, 0.01 M dithiothreitol, and 0.4 M Tris-HCl (pH 8.2). The reduction mixture was cooled to room temperature, and iodoacetic acid was added as a freshly prepared aqueous solution (40 mg/ml) in the amount just needed to alkylate all thiol groups present, including those in the dithiothreitol. Alkylation was allowed to proceed 1 hr in the dark, and then a few crystals of dithiothreitol were added to destroy any excess iodoacetate. When the mixture was dialyzed against cold water, the protein sometimes precipitated and sometimes stayed in solution, and could always be recovered by saturating with ammonium sulfate.

Amino-Terminal Analysis. The enzyme was reacted at room temperature for 30 min with a large excess of dansyl chloride (5 mg/mg of protein), in 0.02 M sodium phosphate (pH 8.2), which was 4 M in urea, 25% by volume dimethylformamide and 10% by volume acetonitrile. The dansylated protein was isolated by precipitation with 10% trichloroacetic acid, washed with acetone, dried *in vacuo*, and hydrolyzed 4 hr at 110° in 6 N HCl. The dried hydrolysate was triturated with 3 drops of ethyl acetate which had been saturated with water just before use, and this solution spotted in the corner of a polyamide layer (Ching Cheng Trading Co., Taiwan) (Woods and Wang, 1967). Chromatograms were developed with 1.5% aqueous formic acid in the first dimension, followed after drying by benzene–acetic acid (9:1) in the second. The identities of the spots observed were confirmed by spotting authentic standards on the developed chromatograms, next to the unknowns, and developing further with the appropriate solvent.

The cyanate procedure was used for quantitation of the amino-terminal residues, exactly according to Stark and Smythe (1963), except that internal standards were included and 2% sodium dodecyl sulfate was used in the carbamylation step instead of urea in some experiments. These modifications were devised by T. Hoffmann (personal communication).

Routine Assay. The enzyme activity was monitored by a modification of the spectrophotometric method used by several others (Mulhausen and Mendocino, 1970). The buffer was 0.010 M Tris-HCl–0.032 M L-histidine (pH 7.4), and reaction mixtures contained 1 mM $MgCl_2$, 50 μg/ml of gelatin, 10 μg/ml of yeast glucose 6-phosphate dehydrogenase (Sigma), 40 μg/ml of $NADP^+$, 0.085 mM glucose 1-phosphate, and 1.6 μM glucose 1,6-diphosphate (all at final concentrations). Reaction volume was usually 1.0 ml. Rates were determined at room temperature (21 ± 1°) by following the absorbance increase at 340 nm in 1-cm path cuvets with a Gilford 2400 recording single-beam spectrophotometer. One unit of enzyme activity is 1 μmole of $NADP^+$ reduced per min under these standard conditions. By assaying a suitable dilution of the enzyme in this assay at 21° and simultaneously by the assay of Ray and Roscelli (1963) at 30°, we find that the activity at 30° is 1.9 ± 0.1 times that at 21°. Therefore enzyme of specific activity 350 U/mg, used in this study, would have a specific activity of 665 U/mg at 30°.

Protein Concentrations. Protein was determined by absorbancy ($E^{1\%}_{1\,cm}$ 7.70 at 278 nm (Najjar, 1955)) in the case of

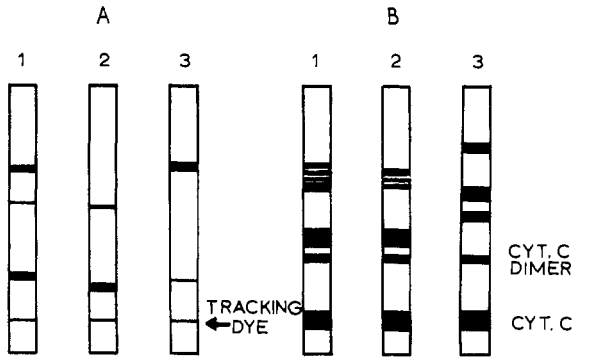

FIGURE 1: Diagrams showing the electrophoresis of the enzyme on polyacrylamide gels. The anode is at the bottom. (A) Under nondenaturing conditions, pH 8.9, by method of Davis (1964), except that no stacking gel was used. From left to right, the samples are: (1) Sigma crystalline phosphoglucomutase as supplied, (2) phosphoenzyme after CM-cellulose chromatography, and (3) dephosphoenzyme after chromatography. (B) In sodium dodecyl sulfate–2-mercaptoethanol, showing from left to right: (1) the phosphoenzyme, (2) the dephosphoenzyme, (3) standard proteins for molecular weight determination. From the bottom up, the standards are: horse heart cytochrome *c* (mol wt 12,400), cytochrome *c* dimer (mol wt 24,800), ovalbumin (mol wt 44,000), catalase (mol wt. 60,000), phosphorylase A (mol wt 94,000).

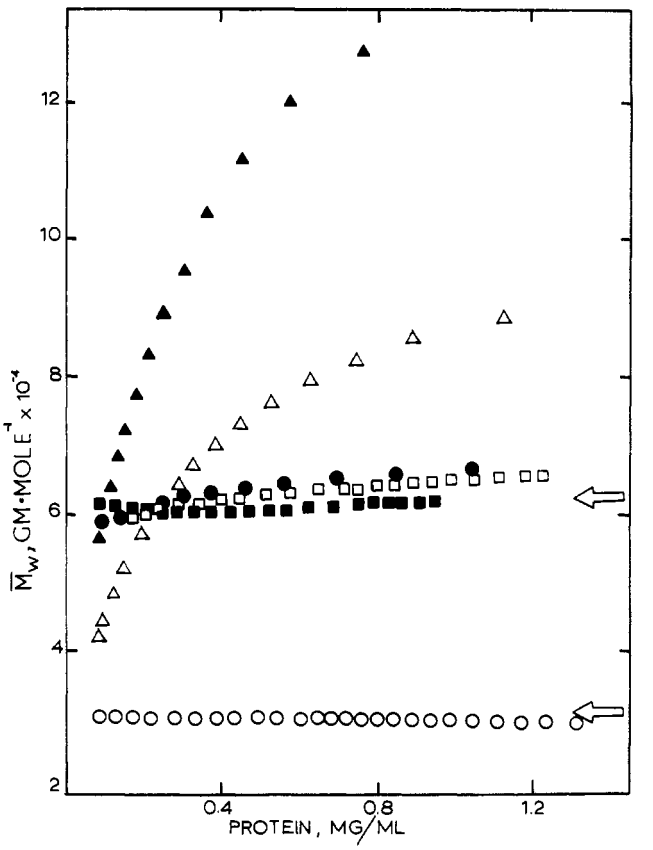

FIGURE 2: Weight-average molecular weight of dephosphoenzyme as a function of protein concentration. The curves are (●) undenatured, pH 7.0; (□) 7.5 M guanidine-HCl–0.1% 2-mercaptoethanol, pH 7.0; (■) same, pH 2.0; (▲) carboxymethyl enzyme, 5.32 M guanidine-HCl–0.1% 2-mercaptoethanol, pH 7.0; (Δ) same, but carboxymethylation conducted in guanidine-HCl + sodium dodecyl sulfate (see text); (○) 0.10% sodium dodecyl sulfate–0.1% 2-mercaptoethanol pH 7.0). The arrows at the right side of the figure are at molecular weight values of 31,000 and 62,000 g/mole.

the native enzyme, and by amino acid analysis or occasionally the procedure of Lowry *et al.* (1951) when the enzyme was denatured.

Results

Gel Electrophoresis of Phospho- and Dephosphoenzyme. Chromatography of Sigma crystalline rabbit muscle phosphoglucomutase (lot 119B-4740) on CM-cellulose (Yankeelov *et al.*, 1964) yielded two peaks of about equal size and specific activity. The phosphoenzyme was eluted first (see Alpers and Lam, 1969). The completeness of the separation was readily demonstrated by electrophoresis through polyacrylamide gels, since phosphoenzyme has a greater mobility in these gels. This is expected because of the extra negative charge acquired by the protein upon phosphorylation. A small amount of phosphoenzyme (less than 10% of the total) remained in the dephosphoenzyme fraction after chromatography. A third, minor protein component was visible on the gels of unchromatographed enzyme, running about half-way between the two phosphoglucomutase bands, and upon chromatography this appeared entirely in the phosphoenzyme fraction.

When the electrophoresis was conducted in gels which contained 0.10% sodium dodecyl sulfate, at pH 7.0, both phospho- and dephosphoenzyme showed a single band, whose molecular weight was slightly greater than 61,000 g/mole to judge from its mobility relative to protein standards of known molecular weights on the gels (Figure 1). Occasionally, when a large amount of the enzyme was loaded on the gels, a trace of material near 30,000 g/mole was also seen. Because of the possibility that remnants of ammonium persulfate in the gels, left over from the polymerization step, could be causing disulfide cross-links and thus preventing dissociation of the molecule, we preran the gels for 2 hr or more at 8 mA/gel to remove any persulfate by electrophoresis. Small ions take about 2.5 hr to move all the way through the gels under these conditions. Loaded on prerun gels, the enzyme then ran as a mixture of components, of which about two-thirds (estimated from optical density scanning of the stained gels) had a molecular weight near 31,000 g/mole, while the rest consisted of three or four discrete bands in the range 65,000–70,000 g/mole. A second method of removing persulfate was also used, in which 0.1% 2-mercaptoethanol was actually included in the gel polymerization mixture. Gel formation was a little slower, but electrophoresis of the enzyme through the resulting gels gave about 90% or more of the 31,000 g/mole material. These results may be taken to mean that persulfate-mediated cross-linking of the enzyme subunits does occur, and that this effect is largely but not utterly abolished by treatments which should remove most persulfate from the gels.

We have recently examined two lots of Boehringer phosphoglucomutase (lots 7311413 and 7311415) by electrophoresis through the dodecyl sulfate gels which had been treated to remove persulfate. In both cases, practically all of the protein (more than 90%) was found at a position corresponding to a molecular weight of about 32,000 g/mole.

Carboxymethylated dephosphoenzyme is almost insoluble in 0.1% sodium dodecyl sulfate solutions, and upon electrophoresis through prerun gels this protein shows some material at an apparent molecular weight of 60,000–70,000 g/mole, and none at any lower molecular weight.

Ultracentrifuge Studies on Dephosphoenzyme. We have confirmed a molecular weight of about 62,000 g/mole for dephosphoenzyme in neutral buffers of moderate ionic

TABLE I: Molecular Weight of Dephosphoenzyme in Various Media (Enzyme Obtained from Sigma).

Sample	Medium Used[a]	$\overline{M_w}$, Overall[b] (g/Mole)	$\overline{M_n}$, Extrapolated[c] (g/Mole)	$\overline{M_w}$, Extrapolated[c] (g/Mole)
PGM[d]	Phosphate (0.01 M) + NaCl (0.1 M), pH 7.0	65,300 ± 700		
PGM	Guanidine-HCl, 7.5 M, pH 7.0	63,400 ± 900		
PGM	Guanidine-HCl, 7.5 M, pH 2.0	60,800 ± 800		
CM-PGM[e]	Guanidine-HCl, 5.32 M, pH 7.0	78,400 ± 1600	31,200	35,800
CM-PGM (+ SDS[f])	Guanidine-HCl, 5.32 M, pH 7.0	61,100 ± 1800	32,450	43,100
PGM	SDS, 0.10%	31,700 ± 400		
PGM	SDS, 0.10%	31,300 ± 300		

[a] All denaturing media (guanidine-HCl and SDS) contained in addition 0.1% 2-mercaptoethanol. [b] This is the average value of $\overline{M_w}$ at the midpoint of the solution column, and refers to an actual protein species only if the sample is homogeneous. [c] Extrapolations performed as explained in footnote 1. [d] PGM = phosphoglucomutase. [e] CM = carboxymethyl. [f] SDS = sodium dodecyl sulfate.

strength (Figure 2) using the high-speed equilibrium technique of Yphantis (1964). Essentially the same value is found in 7.5 M guanidine hydrochloride–0.1% 2-mercaptoethanol, at pH 7 or 2 (Figure 2). The enzyme is insoluble at pH 2 or lower in phosphate buffer. It will be noted that the molecular weights measured have very little concentration dependence, a fact which testifies to the stability and specificity of polypeptide interactions in this enzyme.

Carboxymethylated dephosphoenzyme begins to dissociate to half-molecules at concentrations of guanidine hydrochloride above 5 M. Thus, in 5.32 M guanidine hydrochloride–0.1% 2-mercaptoethanol, a continuous, concentration-dependent distribution of molecular weights is seen (Figure 2), ranging up above 120,000 g/mole. Extrapolation of the weight-average molecular weights to zero concentration[1] yields limiting values near 30,000 g/mole which are listed in Table I, for two different preparations of carboxymethylated dephosphoenzyme. That which dissociated to a greater extent was prepared with a saturating concentration of the dodecyl sulfate present in the guanidine hydrochloride, and traces of bound sodium dodecyl sulfate may have contributed to the dissociation of the protein in the ultrancentrifuge.

By equilibrium dialysis it was found that dephosphoenzyme will bind as much as 0.48 ± 0.05 g of dodecyl sulfate anion per g of protein at 21°, when the concentration of free sodium dodecyl sulfate is 0.10%, in 0.1% 2-mercaptoethanol–0.01 M sodium phosphate (pH 7.0). This value is like those found under similar conditions for other proteins such as bovine serum albumin (Strauss and Strauss, 1958), apoferritin (Hoffman and Harrison, 1963), acetyl-coenzyme A carboxylase (Gregolin *et al.*, 1968), and coliphage φX174 coat protein (Carusi and Sinsheimer, 1963). It is thus unlikely that the molecular weight of the apparent "half-molecules," determined by electrophoresis in the dodecyl sulfate gels, is an artifact caused by an unusually large amount of detergent binding by this protein. Equilibrium centrifugation of preparations of dephosphoenzyme which had been equilibrated in 0.10% sodium dodecyl sulfate–0.1% 2-mercaptoethanol indicated a homogeneous species (see Figure 2), whose molecular weight after correction for the dodecyl sulfate binding as described in the Experimental Section (Carusi and Sinsheimer, 1963) was 31,500 ± 500 g/mole.

These last results were obtained with the Sigma enzyme after diaylzing against the dodecyl sulfate for 48 hr. A second preparation of pure rabbit phosphoglucomutase obtained from W. R. Ray, Jr., of Purdue University, equilibrated more slowly with the dodecyl sulfate. Both phospho and dephospho forms of this preparation were investigated, and were found to behave approximately the same. In Table II are given dialysis times, amounts of the dodecyl sulfate bound, and average molecular weights obtained. Only the samples binding 0.38 g of the dodecyl sulfate/g of protein or more appeared to be homogeneous. These latter samples were submitted to electrophoresis through the dodecyl sulfate gels without any further pretreatment, and were found to consist mostly of material of weight 35,800 ± 1250, with some material at 66,900 ± 1900 g/mole. It seems likely, from these results, that phosphoglucomutase from different sources can vary somewhat in its susceptibility to the dodecyl sulfate, and that all samples of the protein are unusually hard to denature with the detergent.

The samples of reduced, carboxymethylated phosphoglucomutase in guanidine hydrochloride–mercaptoethanol are the only ones examined which show a great heterogeneity in the ultracentrifuge. The data obtained may be further analyzed by use of the two-species plot (Roark and Yphantis, 1968), in which the reciprocal of a given molecular weight average ($\overline{M_w}$, $\overline{M_z}$, etc) is plotted as a function of the reciprocal

[1] These extrapolations were performed by assuming that $1/\overline{M_w}$, the reciprocal weight-average molecular weight, is a cubic function of protein concentration, and fitting the appropriate data in Figure 2 to this function by the method of least squares. Fitting reciprocal molecular weight moments to a series expansion in concentration is a rational procedure, and the range of data available does not justify using a higher order polynomial in the fit. The program of Roark and Yphantis (1968) provides the reciprocal weights as a part of its output.

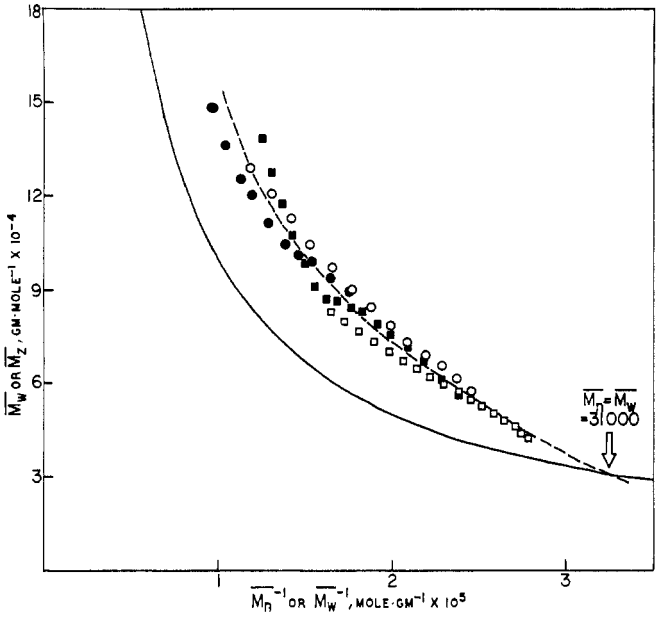

FIGURE 3: Two species plot for carboxymethyl enzyme in 5.32 M guanidine-HCl–0.1% 2-mercaptoethanol (pH 7.0). Symbols are (○) $\overline{M}_w$ *vs.* $\overline{M}_n^{-1}$, carboxymethylation conducted in guanidine-HCl–mercaptoethanol only; (●) $\overline{M}_z$ *vs.* $\overline{M}_w^{-1}$, same sample; (□) $\overline{M}_w$ *vs.* $\overline{M}_n^{-1}$, carboxymethylation in guanidine-HCl–mercaptoethanol + sodium dodecyl sulfate; (■) $\overline{M}_z$ *vs.* $\overline{M}_w^{-1}$, same sample. The solid line is the hyperbola calculated for $\overline{M}_n = \overline{M}_w = \overline{M}_z$, and the broken line is drawn by eye to show the apparent trend of the data. For further details, see text.

of the next lower order average ($\overline{M}_n$, $\overline{M}_w$, etc., respectively). Associating systems which contain only two species (*e.g.*, a monomer and a dimer) yield straight lines in such plots, whose slopes and intercepts are functions of the molecular weights of the two species (Roark and Yphantis, 1968). If the plot obtained is concave up, more than two species are involved, while charge nonideality may cause anomalous plots which are concave down (Roark and Yphantis, 1968). Since the two-species plot is examining the dependence of one molecular weight average upon another, the data for $\overline{M}_w$ *vs.* $\overline{M}_n^{-1}$ will fall on the same curve as those for $\overline{M}_z$ *vs.* $\overline{M}_w^{-1}$. No information is obtained about the dissociation constants for the system under examination.

In Figure 3 is presented a two-species plot combining the data for the two preparations of carboxymethylated dephosphoenzyme whose weight-average molecular weights were shown as a function of concentration in Figure 2. The data for the two samples coincide, a fact which indicates that although the dissociation constants for the samples differ (see Figure 2), no difference is detected between them as regards the types of oligomers formed. Also in Figure 3, we have drawn the hyperbola for $\overline{M}_w = \overline{M}_n$, which will intersect with a two-species plot at any point at which only one molecular weight species exists. The curve defined by the data in Figure 3 would probably extrapolate to intersect with this hyperbola at a point corresponding to the mol wt 31,000 approximately, indicated by the arrow in the figure. This agrees with the other estimates made for the size of the phosphoglucomutase monomers. The general trend of the data toward higher molecular weights suggests that the plot may not intersect with the hyperbola in this direction, that is, that carboxymethylated phosphoglucomutase may polymerize in this medium to an infinite extent. This analysis indicates that when

TABLE II: Sodium Dodecyl Sulfate Binding and Molecular Weight of Phosphoglucomutase (Enzyme Obtained from W. J. Ray).[a]

Enzyme Form	Incubation Time (hr)	DS^- Anion Bound (g/g of Protein)	$\overline{M}_w$, Av (g/Mole)
Dephospho-	60	0.245	41,400
Dephospho-	60	0.284	49,200
Dephospho-	226	0.461	35,350
Dephospho-	226	0.381	35,200
Phospho-	60	0.172	45,850
Phospho-	226	0.387	31,800

[a] Plot $\overline{M}_w$, average, was calculated from a least-square fit to the ln *c* *vs.* $r^2/2$ plot in each case. Heterogeneity could be detected in the samples binding less than 0.3 g of the dodecyl sulfate/g of protein.

dephosphoenzyme is forced to dissociate by carboxymethylation and denaturation with guanidine hydrochloride, the specificity of the monomer–dimer interaction in the native molecule disappears.

End-Group Analysis of Phosphoglucomutase. We have failed to detect any free amino-terminal residues in phosphoenzyme by the dinitrophenylation procedure, using a method that should have detected one-twentieth of a residue per mole of enzyme (62,000 g). This confirms the observations of others (Joshi and Handler, 1964; Milstein and Sanger, 1961; Harshman *et al.*, 1963).

Chromatography of the α-dansylated amino acids obtained by acid hydrolysis of rabbit muscle dephosphoenzyme which had been treated with a large excess of dansyl chloride, however, revealed approximately equal amounts of two components moving in the positions of α,ε-didansyllysine and α-dansylvaline. Little or no α-dansylamino acid was seen when phosphoenzyme was submitted to this procedure. The cyanate procedure was conducted on two batches of dephospho- and one of phosphoenzyme, using 8 M urea or 2% sodium dodecyl sulfate as denaturants during carbamylation. The results are listed in Table III. As will be noted, carbamylation was probably incomplete in all experiments,[2] a fact which is not surprising since the protein precipitated from the reaction medium soon after cyanate was added. From Table III it can be seen that phosphoenzyme yielded no amino-terminal acid in stoichiometric amounts, while dephosphoenzyme gave about 1 mole of valine per 62,000 g, but no lysine.

One experiment, in which phosphoenzyme (7.8 mg) was subjected to hydrazinolysis for 20 hr in anhydrous hydrazine, yielded only traces of amino acids on the amino acid analyzer (less than 0.1 mole of any amino acid per 62,000 g of protein). Attempts to release carboxyl-terminal residues from dephosphoenzyme with carboxypeptidases A and B, either singly or together, have also failed. This last result agrees with earlier observations on the phosphoenzyme (Harshman *et al.*, 1963).

[2] When totally carbamylated proteins are hydrolyzed in 6 N HCl, the percent of lysine plus homocitrulline which is recovered as lysine is in the range 17–30% (Stark and Smythe, 1963). Higher values than this presumably arise if carbamylation is incomplete.

TABLE III: Determination of Amino-Terminal Residues in Phosphoglucomutase by the Cyanate Procedure.

Enzyme Form	Denaturant during Carbamylation	Enzyme Taken (μmole)	N-Terminal Amino Acids Found (Moles/Mole)			% of[b] Lys + Hci[c] Recovd as Lys
			Val	Lys	Others[a]	
Dephospho-	2% SDS[d]	0.226	0.90	0.041	Glutamate (0.26), glycine (0.25)	40.2
Dephospho-	6 M Urea	0.045	1.09	0.086	Glutamate (0.41), glycine (0.74), alanine (0.35)	37.9
Phospho-	2% SDS	0.204	0.15	0.062	Glutamate (0.48)	34.3

[a] Additional amino acids detected at greater than 0.2 mole/mole of the enzyme. Glutamate and glycine are common contaminants in the procedure. We have observed similar levels of contamination with other proteins—bovine hemoglobin and pancreatic ribonuclease A—and therefore do not regard them as evidence for heterogeneity of the enzyme under study. No N-terminal amino acid apart from lysine and valine was seen in the dansyl procedure. [b] This percentage measures the degree of carbamylation achieved. See footnote 2. [c] Hci = homocitrulline. [d] SDS = sodium dodecyl sulfate.

Discussion

The results presented here provide strong evidence for the belief that rabbit muscle phosphoglucomutase is a very stable dimer of two subunits. These subunits are of equal size, within the limits of precision of the measurements performed, and appear to possess different amino terminal residues, namely, valine and lysine. That the subunits, if they existed, would have to be chemically different was predicted from earlier peptide mapping work (Joshi *et al.*, 1967; Joshi and Handler, 1964, 1969). An indication of the strength of the forces which bind these half-molecules together is the fact that guanidine hydrolchloride cannot dissociate them at all, although the dodecyl sulfate is a good denaturant for the molecule.

Dephosphoenzyme contains one amino-terminal residue, valine, which can be made available for carbamylation with cyanate, while the second residue, lysine, is detected only by the dansyl procedure. It may be that this lysyl residue is located in a part of the molecule in which the aromatic reagent, dansyl chloride, can dissolve while the ionic one, cyanate, cannot. In phosphoenzyme, neither amino-terminal residue is available for carbamylation or dansylation. Further dinitrophenylation experiments, on the dephosphoenzyme, might confirm that lysine is an amino terminal residue, but we have not explored this.

It was useful to choose the dephosphoenzyme for the studies reported here. Carboxymethylated phosphoenzyme does not dissociate at all in 5 M guanidine hydrochloride–mercaptoethanol, according to Harshman and Six (1969), while 5.32 M of the same reagent effects considerable dissociation of the carboxymethylated dephosphoenzyme in our hands. The monomers are remarkably hard to separate, although the forces of interaction are completely noncovalent as far as can be seen. None of the cysteinyl residues in rabbit muscle phosphoglucomutase participate in the formation of a disulfide bond (Bocchini *et al.*, 1967). The different susceptibility of phospho- and dephosphoenzymes to denaturation, and the different reactivity of the amino-terminal residues in the two enzyme forms, may be added to the list of changes seen when rabbit muscle phosphoglucomutase is phosphorylated or dephosphorylated. These changes presumably reflect a striking conformational rearrangement within the protein, accompanying phosphorylation, which thus is an important feature of the catalytic process in this enzyme.

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References

Alpers, J. B., and Lam, G. K. H. (1969), *J. Biol. Chem. 244*, 200.

Bocchini, V., Alioto, M. R., and Najjar, V. A. (1967), *Biochemistry 6*, 313.

Carusi, E. A., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 338.

Cohn, E. J., and Edsall, J. T. (1943), *in* Proteins, Amino Acids and Peptides, Cohn, E. J., and Edsall, J. T., Ed., New York, N. Y., Reinhold, p 370.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Filmer, D. L., and Koshland, D. E., Jr. (1963), *Biochim. Biophys. Acta 77*, 334.

Gregolin, C., Ruder, E., Warner, R. C., Kleinschmidt, A. K., and Lane, M. D. (1968), *J. Biol. Chem. 243*, 4236.

Harshman, S., Robinson, J. P., and Najjar, V. A. (1963), *Ann. N. Y. Acad. Sci. 103*, 688.

Harshman, S., and Six, H. R. (1969), *Biochemistry 8*, 3423.

Hashimoto, T., and Handler, P. (1966), *J. Biol. Chem. 241*, 3940.

Hoffman, T., and Harrison, P. M. (1963), *J. Mol. Biol. 6*, 256.

Hooper, J., Joshi, J. G., Sakurada, T., Kuwaki, T., Swanson, J. R., and Handler, P. (1968), *Fed. Proc. 27*, 639.

Joshi, J. G., and Handler, P. (1964), *J. Biol. Chem. 239*, 2741.

Joshi, J. G., and Handler, P. (1969), *J. Biol. Chem. 244*, 3343.

Joshi, J. G., Hooper, J., Kuwaki, T., Sakurada, T., Swanson, J. R., and Handler, P. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1482.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Milstein, C. P., and Milstein, C. (1968), *Biochem. J. 109*, 93.

Milstein, C., and Sanger, F. (1961), *Biochem. J. 79*, 456.

Mulhausen, H., and Mendocino, J. (1970), *J. Biol. Chem. 24*, 4038.

Najjar, V. A. (1955), *Methods Enzymol. 1*, 294.
Najjar, V. A. (1962), *Enzymes 6*, 161.
Najjar, V. A., and Pullman, M. E. (1954), *Science 119*, 631.
Peck, E. J., Jr., Kirkpatrick, D. S., and Ray, W. J., Jr. (1968), *Biochemistry 7*, 152.
Ray, W. J., Jr., and Roscelli, G. A. (1964), *J. Biol. Chem. 239*, 1228.
Roark, D. E., and Yphantis, D. A. (1968), *Ann. N. Y. Acad. Sci. 164*, 245.
Robinson, J. P., and Najjar, V. A. (1961), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 20*, 90.
Stark, G. R., and Smythe, D. G. (1963), *J. Biol. Chem. 238*, 214.
Strauss, G., and Strauss, U. P. (1958), *J. Phys. Chem. 62*, 1321.
Weber, K., and Osborn, J. (1969), *J. Biol. Chem. 244*, 4406.
Woods, K. R., and Wang, K. (1967), *Biochim. Biophys. Acta 133*, 369.
Yankeelov, J. A., Jr., Horton, H. R., and Koshland, D. E., Jr. (1964), *Biochemistry 3*, 349.
Yankeelov, J. A., Jr., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240*, 1593.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Selective Modification of the Mitochondrial Isozyme of Aspartate Aminotransferase by β-Bromopropionate. I. Inactivation Process and Properties of Inactivated Enzyme†

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ABSTRACT: A comparative study on the inactivation of the phosphopyridoxal forms of the mitochondrial and supernatant isozymes of aspartate aminotransferase from pig heart muscles by various halo acids (iodoacetate, iodoacetamide, α-bromopropionate, β-bromopropionate, γ-bromobutyrate, and bromosuccinate) was conducted. Of these halo acids, β-bromopropionate was found to cause an irreversible inactivation of the mitochondrial enzyme. None of them inactivated the supernatant enzyme. The inactivation of the mitochondrial enzyme followed pseudo-first-order kinetics. The dependence of the inactivation rate on the concentration of β-bromopropionate showed typical saturation kinetics, indicating substrate-like structural features of this reagent. The maximum rate constant for inactivation was 0.4 hr^{-1} at 25° and the constant analogous to the Michaelis constant for β-bromopropionate was calculated to be 100 mM which was of a magnitude similar to that of the related constant found when the halo acid acted as competitive inhibitor in the transamination reaction. The presence of maleate or succinate protected the enzyme from the inactivation. The inactivation rate increased with increasing pH up to 7, above which the rate remained unchanged. The pK for the inactivation reaction was about 6.2. As inactivation progressed, a new peak appeared around 395 nm with a concomitant decrease in the 355-nm peak of the native enzyme. The new peak at 395 nm was not changed upon variation of pH or addition of substrates. Furthermore, the 395-nm band did not disappear upon the addition of $NaBH_4$ under the condition where the internal aldimine bond of the native enzyme was readily reduced. The 395-nm absorption band was accompanied by a negative circular dichroism band at 410 nm. The amount of bound pyridoxal phosphate did not change during the inactivation. The number of sulfhydryl groups of the enzyme was not affected by the modification. Amino acid analysis of the inactivated preparation demonstrated N^{ϵ}-(2-carboxyethyl)lysine as a major product of alkylation of the enzyme by β-bromopropionate, along with two minor products derived from histidine. From a quantitative correlation of the extent of inactivation with the amount of carboxyethylated amino acid residues, it was concluded that modification of one lysine residue per monomeric unit of the enzyme was responsible for the inactivation.

Two distinct forms of L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) are present in mammalian tissues, one of which is localized in mitochondria and the other in the soluble cytoplasmic fraction (Boyd, 1961; Katsunuma *et al.*, 1962). A number of investigations (Morino *et al.*, 1963, 1964; Morino and Wada, 1963; Wada and Morino, 1964; Wada *et al.*, 1966; Jenkins and D'Ari, 1966; Martinez-Carrion and Tiemeier, 1967; Kagamiyama *et al.*, 1968; Michuda and Martinez-Carrion, 1969, 1970; Morino and Watanabe, 1969; Morino and Okamoto, 1970) have revealed that these two are differentiated from each other by kinetic, physicochemical, and immunochemical properties.

The similarities in the mode of action and the apparently clear structural distinction prompt a comparative study of the chemical nature of the active site in each isozyme. As an approach to this aim, we studied the primary structure of the tetrapeptides at the pyridoxal phosphate binding site obtained from chymotryptic digests of both enzymes. The two peptides are quite similar as indicated by the conservative substitution of amino acid residues at the corresponding positions with a sequence, Lys-Asn, common to both (Morino and Watanabe, 1969). Thus it has been anticipated that both isozymes should have essential common structural features at least in their active region.

To learn more about the chemical structure of the catalytic

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