Conformational States of Native and Denatured Phosphoglucose Isomerase. I. Titration of Exposed and Buried Amino Acid Residues*

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ABSTRACT: Rabbit muscle phosphoglucose isomerase, in the native state and under various conditions of denaturation, was subjected to electrometric and spectrophotometric titration with the object of determining the number of exposed and buried histidyl and tyrosyl residues and of investigating their possible role in maintaining the conformation of the enzyme. In native phosphoglucose isomerase, 20 of the total of 47 histidyls and all of the 24 tyrosyls are inaccessible. Acid denaturation normalizes the titration of the 20 anomalous histidyls, and a strongly alkaline medium that of all of the tyrosyls. Sodium dodecyl sulfate (0.5%) allows all of the 12 sulfhydryl groups to be titrated but does not expose any of the tyrosyls, whereas 8 M urea brings all 24 of them into contact with the solvent. The parallel nature of enzyme activity loss and exposure of about 20 histidyls and 20 tyrosyls, on acid or alkaline denaturation, is interpreted in terms of their participation in maintaining the native structure of phosphoglucose isomerase through formation of hydrogen bonds which are stabilized by hydrophobic shielding from competition by water molecules.

On the basis of the electrometric titration studies, average intrinsic pK values for the ionization of lysine (10.2) and of imidazole (6.9) and carboxyl groups (4.2) were calculated. In addition, a detailed study of the electrophoretic mobility as a function of the ionic strength yielded an isoelectric point of 8.5 at 1° when extrapolated to zero ionic strength. The isoionic point was found to be 7.47 by direct measurement at 30°.

Amino acid analysis of rabbit muscle phosphoglucose isomerase (D-glucose 6-phosphate ketolisomerase, EC 5.3.1.9) has shown that the enzyme contains 47 histidine and 24 tyrosine residues per molecule of 132,000 molecular weight (K. D. Schnackerz and E. A. Noltmann, unpublished experiments). Both tyrosine and histidine may participate in hydrogen bonding involved in maintaining the structural integrity of the native enzyme molecule, and their effectiveness in this respect will be significantly increased if the bonds are screened from interaction with the solvent by the protein matrix (Scheraga, 1963). In addition, both types of residues, but more especially tyrosine, may contribute by their ability to form hydrophobic bonds (Scheraga, 1963). It is, therefore, important to know the extent to which the histidine and tyrosine residues of native phosphoglucose isomerase in solution are screened from the medium, especially in view of the unusually high histidine content of this enzyme.

This paper is concerned with electrometric and spectrophotometric titrations of rabbit muscle phosphoglucose isomerase, and with experiments on free-boundary electrophoresis. The titration methods have been used to obtain quantitative data on the number of histidine and tyrosine residues available to the solvent in the native molecule. In addition, the exposure of buried histidine and tyrosine residues during acid or alkaline denaturation has been used as a probe of conformational changes occurring during that process.

In a previous paper (Dyson and Noltmann, 1968) kinetic evidence was presented to show that the catalytic activity of muscle phosphoglucose isomerase was dependent upon two ionizable groups with pK values of 6.75 and 9.30 (at 30°). A further object of the present study was to determine whether conformational changes were detectable at hydrogen ion concentrations corresponding to the measured pK values, which might be related to the ionization of the groups concerned. This was necessary in order to establish whether these groups were directly involved in the catalytic process, or were required to maintain the active conformation of the molecule.

Materials and Methods

Enzyme Preparation. Phosphoglucose isomerase was isolated from rabbit skeletal muscle according to the previously described method (Noltmann, 1964, 1966). All of the preparations used in the present work were five times crystallized and had specific activities of 800–900 μmoles of fructose 6-phosphate converted into glucose 6-phosphate per min at 30°. Prior to each series of experiments, an aliquot of the crystalline suspension was centrifuged at 0° for 45 min at 27,000g. The pellet was dissolved in the same medium used subsequently for dialysis, which was conducted as described for the individual methods. Proteins concentrations were determined spectrophotometrically at 280 mμ with use of an absorptivity coefficient of 1.32 (10-mm light path) for a solution containing 1 mg of enzyme/ml (Noltmann, 1966). In calculations involving molar concentrations of the enzyme, a molecular weight of 132,000 was employed for rabbit muscle phospho-

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glucose isomerase (N. G. Pon, M. N. Blackburn, G. C. Chatterjee, and E. A. Noltmann, unpublished experiments).

Preparation of Isoionic Protein. To obtain the enzyme at its isoionic pH, the crystalline pellet, after centrifugation, was dissolved in distilled water and dialyzed, under nitrogen atmosphere, against a 1000-fold volume of distilled, CO_2-free water. After dialysis it was passed twice through mixed-bed resin (Amberlite MB-3, A. R. Mallinckrodt) (Dintzis, 1952). Subsequent treatment is described for each individual method.

For determination of the isoionic point an aliquot was transferred to a Radiometer TTA 31 microtitration vessel (with water jacket for temperature control) immediately after passage through the resin and kept under nitrogen atmosphere for 2 hr. Measurements of the pH were made under continuously renewing nitrogen atmosphere (see below) either with a Radiometer TTT-1c pH meter equipped with a PHA 630-Ta scale expander or with a Beckman Model 1019 research pH meter: both instruments permit pH readings to 0.001 unit. Before each set of measurements the respective pH meter was standardized with National Bureau of Standards phosphate buffer (0.025 *m* KH_2PO_4–0.025 *m* Na_2HPO_4, pH 6.853, at 30°).

Free-Boundary Electrophoresis. Measurements of the electrophoretic mobility were made at 4.0° in a Perkin-Elmer Model 238 electrophoresis apparatus by photographing, at defined time intervals, schlieren images of the moving boundary. Sample solutions, containing 1–4.3 mg of phosphoglucose isomerase per ml, were prepared by dialyzing suitable enzyme aliquots for a total of 48 hr against at least two changes of a 1000-fold volume each of the desired buffer. Buffers were normally 0.05 M (with the exception of those used for experiments at 0.025 ionic strength for which they were 0.01 M); KCl was added to bring the ionic strength to the desired value, calculated in each case for the degree of dissociation and the p*K* of the corresponding buffer species (at a temperature of 4°). Measurements of the pH were made at 1° with a Beckman Model 1019 research pH meter, standardized with NBS phosphate buffer of pH 6.977 at 1°, as described above. Conductivity measurements were made, also at 1°, with a Radiometer Type CDM-2d conductivity meter equipped with a CDC 104 conductivity cell.

Electrometric Titration. Protein titrations were performed at 30° in a continuous manner (Nozaki and Tanford, 1967) with a Radiometer Titrator (TTT 1c)–Titrigraph (SBR 2c) combination. Wide-range hard-glass electrodes (Radiometer Type B) were used for the titrations and Na^+ was excluded from all solutions to avoid "sodium error." The system was checked before each experiment for linearity and calibrated with standard buffers obtained from the National Bureau of Standards (potassium hydrogen phthalate, pH 4.012, at 30°; borax, pH 9.139, at 30°). Temperature control was maintained to ±0.1° by circulating water through the jacket compartment of the titration vessel (Radiometer TTA 31).

KOH titrant (0.04–1.0 N), prepared with deionized, doubly glass-distilled water that had been boiled (to remove dissolved CO_2) and then cooled under nitrogen, was standardized against potassium hydrogen phthalate before each experiment. Air was passed through a CO_2 absorbant (Mallcosorb, 30–50 mesh, Mallinckrodt) before entering the KOH reservoir in order to prevent the formation of potassium carbonate. HCl titrant (0.04–1.0 N) was analogously standardized against standard KOH. KCl was added to both acid and base titrants to adjust their ionic strength to the same value as that of the protein solution to be titrated. The titrant was delivered into the protein solution with a precision bore, all-glass syringe of 0.5-ml capacity ("Agla," Burroughs Wellcome and Co., London). The combined delivery system was calibrated against pen deflection and chart speed of the recorder; correlation to better than 0.5% was obtained.

For a titration experiment an aliquot of isoionic protein was dialyzed against CO_2-free KCl of the desired ionic strength. Prior to the titration the protein solution was kept for 2 hr under nitrogen atmosphere, and nitrogen was continuously passed over the solution during the titration, which was started 30 min after transfer of the protein to the titration vessel. To avoid evaporation, the nitrogen was bubbled through aqueous KCl of the same ionic strength as that of the protein solution before passing into the titration vessel. Titrations were started from approximately 1 pH unit above, or 1 pH unit below, the isoionic point of the protein, for acid or alkaline titrations, respectively. Superimposed portions in the central pH region of different titration curves were thus available for reference purposes. Each titration was followed by a blank titration over the same pH region with the final KCl dialysate serving as the blank solution. The blank titration was corrected for the smaller volume of titrant added as compared with the protein titration. The same precautions with respect to CO_2 exclusion were taken for the blank as for the protein titration. A net titration curve was constructed for phosphoglucose isomerase by subtracting the curve obtained for the KCl dialysate from the curve obtained for the protein solution.

Spectrophotometric Titration. EXPERIMENTAL CONDITIONS. Absorbance measurements were made in a Cary 15 spectrophotometer equipped with thermostatted cuvet holder. All of the data were obtained by difference spectrophotometry with suitable additions of solvent to the reference cuvet to maintain a protein concentration identical with that in the sample cuvet. Temperature control was maintained to within ±0.1°. Stoppered silica cuvets (cell code S18-360, Pyrocell Manufacturing Co., Westwood, N. J.) with 10-mm light path, matched to a tolerance of 0.001 *A* at 235 mμ, were used for all spectrophotometric measurements. The pH measurements were made in the cuvet with a Beckman Model 1019 research pH meter equipped with a wide-range, hard-glass, semimicro combination electrode (A. H. Thomas Co., Philadelphia, Pa.).

The time dependence of spectral changes was studied as follows. A stopwatch was started simultaneously with addition of titrant to the enzyme. The solution in the cuvet was mixed, the absorbance scan was started, and the elapsed time was noted whenever the scan passed through a wavelength of interest. The scan was repeated as often as necessary, the time elapsed since addition of the titrant being noted as before. ΔA values were plotted as a function of time, in a semilogarithmic manner, and extrapolated to zero time. Equilibrium values for ΔA were obtained from solutions incubated in the cuvet at 30° for 24 hr.

Protein solutions for spectrophotometric titration were prepared in an analogous manner to those for electrometric titration, with the exception that the period under nitrogen atmosphere before titration was omitted.

Spectrophotometric Titration. SELECTION OF WAVELENGTHS FOR STUDY. The contributions to the protein ultraviolet spectrum arising from the ionization of tyrosine and cysteine residues and from conformational changes in the protein molecule

overlap in certain areas. The wavelengths at which to study these three parameters were therefore chosen such that contributions from sources other than the one of immediate interest were at a minimum. When necessary, contributions from other sources were determined, as described below, and subtracted from the total ΔA. The changes in the ultraviolet spectrum of the protein, which arise from the blue shifts occurring when previously screened aromatic amino acid residues (mainly tyrosine and tryptophan) become exposed to the solvent (Wetlaufer, 1962; Herskovits, 1967), have been used to evaluate the extent of the conformational changes occurring during pH-induced denaturation.

The wavelengths selected for the determination of the titration curves of tyrosine and cysteine, and for the study of the extent of the pH-induced conformation changes, were as follows. (1) Ionization of tyrosine phenolic hydroxyl groups was measured at 300 mμ where absorbance contributions from conformational changes are insignificant.[1] (2) Ionization of cysteine sulfhydryl groups was measured at 245 mμ. This wavelength was chosen in order to avoid interference from the ionization of imidazole groups (maximum at 226 mμ) since rabbit muscle phosphoglucose isomerase contains 47 histidine residues (K. D. Schnackerz and E. A. Noltmann, unpublished experiments; Table I of this paper). Absorbance differences due to conformational changes are also negligible at 245 mμ.[1] Corrections for absorbance contributions from tyrosine ionization, when necessary, were made analogously as described under part 3 for corrections at 285 mμ, except that a factor of 5.33 $\pm$ 0.04 for the ratio $\Delta A_{245}/\Delta A_{300}$, obtained with *N*-acetyl-L-tyrosine, was used. (3) Conformational changes at alkaline pH were estimated by measuring the absorbance at 285 mμ and subtracting the absorbance contribution arising from the ionization of phenolic hydroxyl groups.[1,2] This contribution was calculated from a difference spectrum determined for *N*-acetyl-L-tyrosine at pH 11.7 *vs.* 6.7. A value of 0.47 $\pm$ 0.01 was calculated for the ratio $\Delta A_{285}/\Delta A_{300}$ from this difference spectrum. By multiplying the ΔA at 300 mμ (due only to ionization of tyrosine residues at this wavelength) with this ratio, the contribution due to ionization of tyrosine residues at 285 mμ was obtained. This was then subtracted from the total ΔA at 285 mμ to give the contribution produced by the conformational change alone (Donovan, 1964). Both here, and in part 2 above, a correction of 1 mμ was applied in measuring the given ratios, in order to allow for the blue shift of the ΔA maxima in *N*-acetyl-L-tyrosine as compared with the maxima in the protein (Donovan, 1964). (4) A series of acid difference spectra were obtained at gradually decreasing pH. Conformational changes at acid pH were estimated from these spectra by measurement at 292 mμ.[1,2] No ionizations occur at acid pH, which contribute at this wavelength.

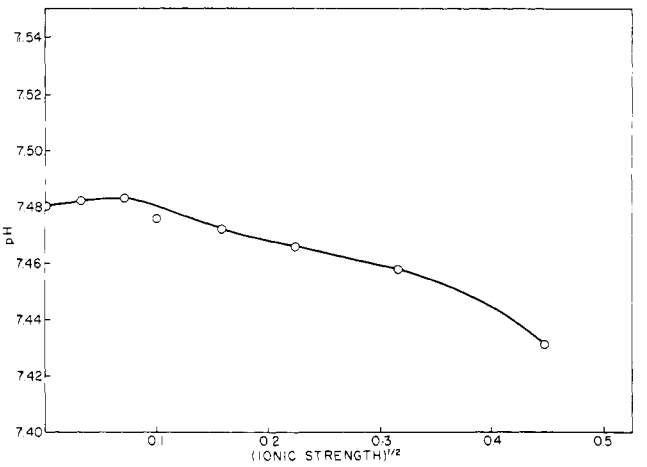

FIGURE 1: Effect of KCl on the isoionic pH of rabbit muscle phosphoglucose isomerase. Protein solution prepared as described under Methods; concentration, 8.6 mg/ml (6.6 $\times$ 10^{-5} M); temperature, 30.0°.

Reagents. Analytical grade urea (Mallinckrodt) was recrystallized three times from 50% aqueous methanol and stored dry at −10°; fresh solutions were prepared immediately before use. Sodium dodecyl sulfate (Matheson Coleman and Bell) was of the highest purity available and was used without further treatment. *N*-Acetyl-L-tyrosine was obtained from Calbiochem. All other chemicals were of the best grade commercially available.

Results

Isoionic and Isoelectric Points. The isoionic point of rabbit muscle phosphoglucose isomerase was found to be at pH 7.47 $\pm$ 0.01 (mean of four determinations) by direct measurement at 30° of the isoionic protein (8–10 mg/ml, prepared as described under Methods). When the pH measurements on the isoionic protein were performed in the presence of increasing concentrations of KCl (Figure 1), a slight decrease in pH occurred reaching about 0.03 pH unit at an ionic strength of 0.2. Since an increase in pH with increasing ionic strength is considered to result from binding of anions, and a decrease from binding of cations (Scatchard and Black, 1949), Cl^- binding apparently does not take place, but the possibility of K^+ binding cannot be entirely excluded. If K^+ binding does occur, it must be to a very small extent, since much larger pH changes have been observed in cases where ion binding was conclusively shown to be present (Nozaki *et al.*, 1959). Attempts to measure the isoionic point at 1° for comparison with the isoelectric point at zero ionic strength (*vide infra*) were unsuccessful, since it was impossible to obtain stable pH readings at this temperature and ionic strength.

A detailed study was made of the electrophoretic behavior of phosphoglucose isomerase as a function of pH, ionic strength, and buffer species. At pH values of between 6.5 and 9.5, the enzyme migrated as a single peak in both the ascending and descending directions. In more acidic and more alk-

[1] See Figure 3 of the accompanying paper (Dyson and Noltmann, 1969).

[2] The peak at 292 mμ arises from changes in the environment of tryptophan only, that at 285 mμ from changes in the environment of both tryptophan and tyrosine residues. Spectra obtained at various stages of pH-induced denaturation indicated that, under the conditions employed, both peaks developed in an essentially parallel manner; either peak could, therefore, be used as a measure of the extent of the conformational change induced by acid or alkaline conditions (*cf.* Figure 3 of the accompanying paper (Dyson and Noltmann, 1969)). The peak at 285 mμ was used in the alkaline region, since the contribution to total ΔA from tyrosine ionization is substantially lower at that wavelength than at 292 mμ. At acid pH, however, where contributions from sources other than environmental changes are absent, the peak at 292 mμ was employed in estimating the conformational change because it has a more pronounced maximum.

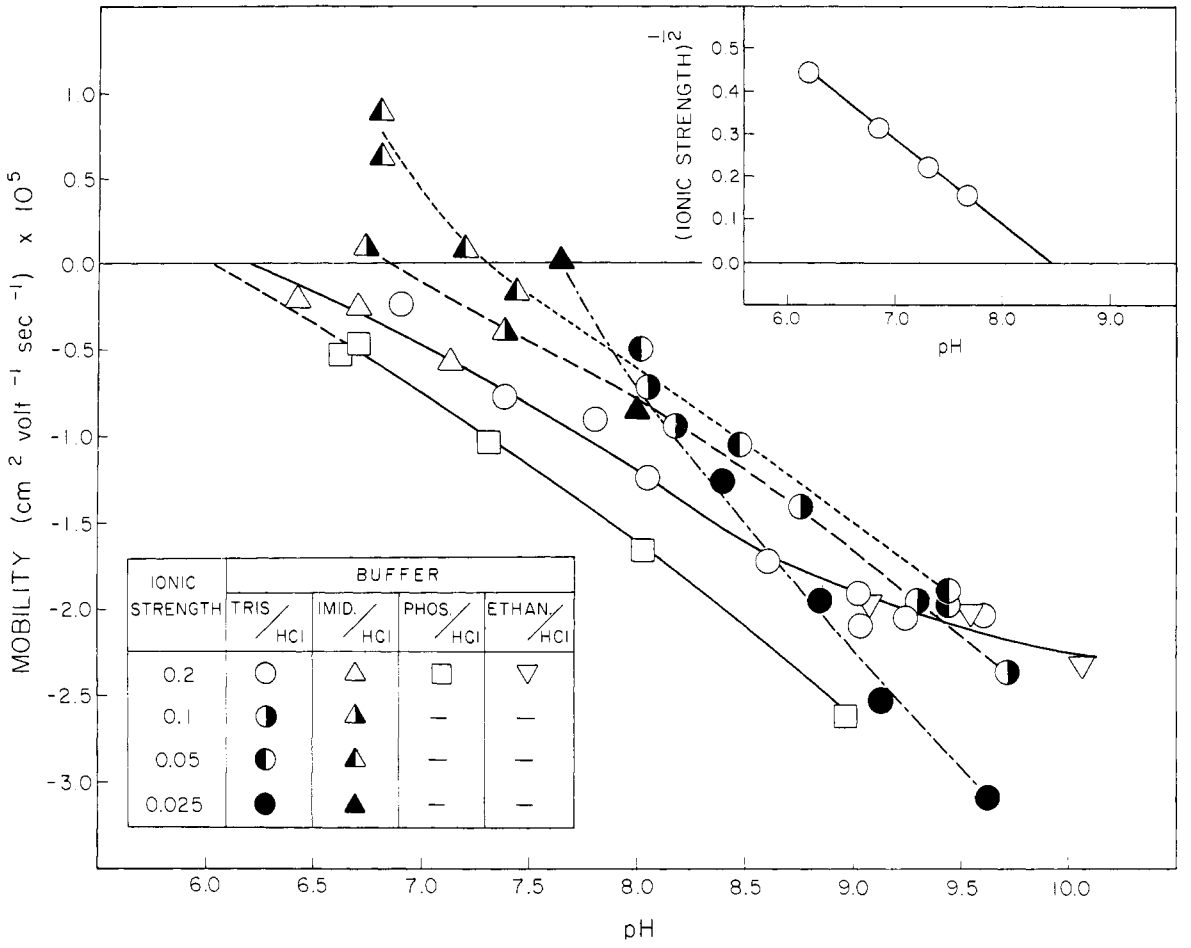

FIGURE 2: Electrophoretic mobility of rabbit muscle phosphoglucose isomerase as a function of pH and ionic strength. The various symbols denote the buffer systems and their ionic strengths as tabulated in the insert in the lower left-hand corner of the figure; 0.05 M buffers were used for ionic strengths of 0.05, 0.1, and 0.2, and 0.01 M buffer for 0.025 ionic strength. KCl was added to adjust to the desired final ionic strengths calculated for pK values and buffer dissociations at 4°, the temperature at which the mobility measurements were made. Conductivity and pH of the buffer solutions were determined at 1° as described under Methods. The curves are drawn as the best visual fit to points representing mobilities at the same ionic strength. The insert in the upper right-hand corner shows the dependence of the isoelectric point upon ionic strength, plotted as the square root of the ionic strength (*ordinates*) upon the isoelectric pH (*abscissa*). Protein concentrations ranged from 1.0 to 4.3 mg per ml; no effect of protein concentration was appararent during the experiments.

aline media, additional minor components were occasionally apparent. These are assumed to be probably the result of a beginning denaturation of the enzyme, since a decrease in enzyme activity was found to accompany such nonideal boundary development.[3]

Electrophoretic mobilities of descending boundaries are summarized in Figure 2, where data corresponding to the same ionic strength are plotted together. For univalent buffers, the isoelectric point is a linear function of the square root of the ionic strength (see upper right-hand insert in Figure 2); such a relationship has been observed for a number of other proteins (*e.g.*, Brown and Timasheff, 1959; Yue *et al.*, 1967a,b). From this graph an extrapolated value of 8.5 at zero ionic strength and 1° may be estimated for the isoelectric point of rabbit muscle phosphoglucose isomerase.

[3] During the preparation of the protein solutions for electrophoretic mobility measurements the protein was exposed to the experimental pH for periods of up to 72 hr. Thus, at pH values toward the limits of stability of the enzyme denaturation may begin to occur.

Values for the mobilities obtained in different univalent buffers at the same ionic strength lie on a smooth curve indicating the absence of significant interaction between these buffer ions and the enzyme; this is in accord with previous kinetic studies of the catalyzed reaction (Dyson and Noltmann, 1968). In contrast, phosphate buffer produced at all pH values increased mobility toward the anode, suggesting interaction between the protein and the buffer species. In agreement with this observation, kinetic studies have shown phosphate to be a weak inhibitor of phosphoglucose isomerase (Salas *et al.*, 1965; Dyson and Noltmann, 1968). Similar increased mobilities have also been found for glucose 6-phosphate dehydrogenase and nucleoside diphosphokinase (Yue *et al.*, 1967a,b).

Hydrogen Ion Titration Curves of Phosphoglucose Isomerase. Composite experimental titration curves, obtained with native and acid-denatured rabbit muscle phosphoglucose isomerase at 0.05 and 0.2 ionic strength, are shown in Figure 3. Each of the curves was constructed as described under Methods from data of the titration experiments indicated in the legend to the figure. Titration curves for the native enzyme were found to

TABLE I: Ionizable Amino Acid Side Chains in Native and Denatured Rabbit Muscle Phosphoglucose Isomerase.

Ionizable Group or Side Chain	Available in the Native Molecule	Titrated in the Denatured Molecule	Determined by Amino Acid Anal.[a]
	Amino Acid Residues or Groups/Enzyme Molecule[b]		
Carboxyl	127	127[c]	135
Total acid binding		170[c]	172
Histidine	27[e]	47[c]	47
Tyrosine	0[d]	24[d]	24
Lysine + arginine	123	123[e]	124
Cysteine	4–8[f]	12[d,f]	12

[a] K. D. Schnackerz and E. A. Noltmann, unpublished experiments. [b] Based on a molecular weight of 132,000 (N. G. Pon, M. N. Blackburn, G. C. Chatterjee, and E. A. Noltmann, unpublished experiments). [c] From Figure 3, calculated as described in the text. [d] From spectrophotometric titration data described in the text. [e] By difference, total acid binding minus histidine content. [f] From Chatterjee and Noltmann (1967).

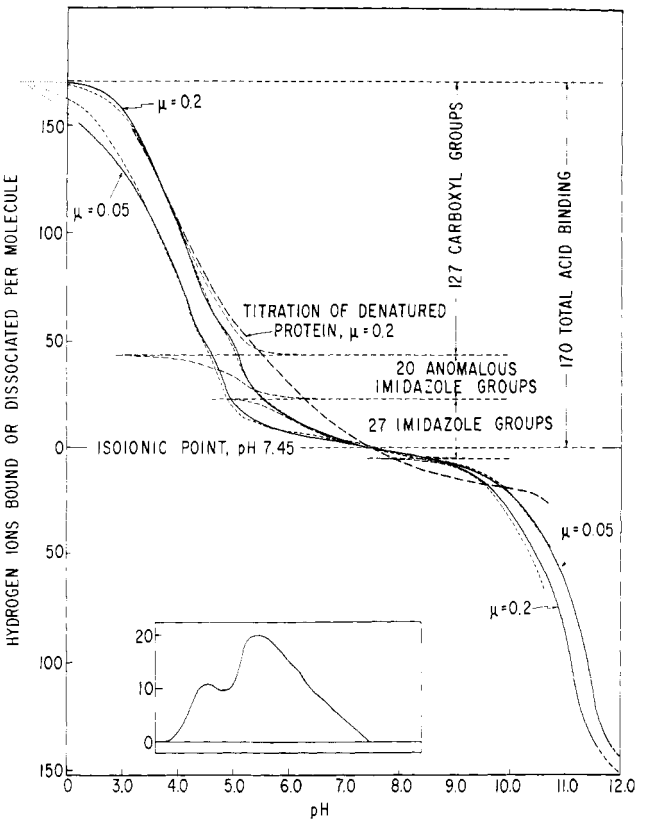

FIGURE 3: Titration curves of rabbit muscle phosphoglucose isomerase. Hydrogen ions bound or dissociated are given on the ordinate with the isoionic point as reference. Titrations were performed at 30° and at either 0.05 or 0.2 ionic strength, as indicated. The experimental curves shown are composites of the following titrations: at 0.2 ionic strength, five acid and four alkaline titrations; at 0.05 ionic strength, three acid and two alkaline titrations; back-titration, two measurements. The continuous curves denote the experimental titration curves obtained as described under Methods, the accompanying light, broken curves are the respective theoretical titration curves calculated from the data on Table I (column 3) and Table III. The heavy, broken curve denotes the experimental titration curve obtained on back-titration of the acid-denatured enzyme at 0.2 ionic strength. The inset in the lower half of the figure represents the difference between the number of groups titrated at the respective pH values in the forward titration (*i.e.*, on addition of acid) and those titrated on back-titration, after acid denaturation of phosphoglucose isomerase.

be fully reversible only between the limits of pH 6 and 10, under the conditions of titration. For an approximate count of the numbers of the different types of groups titrated, the curve obtained at 0.2 ionic strength was divided into three sections, an acid (pH 1.5–6.0), a neutral (pH 6.0–8.5), and an alkaline region (pH 8.5–13), corresponding to the titration of carboxyl groups, imidazole groups, and ϵ-amino, phenolic hydroxyl, sulfhydryl, and arginine groups, respectively. A final determination of the number of each type of group titrated was made by successive fitting of calculated titration curves to the experimental curve, amending the approximate counts as necessary, until the best fit was obtained, in the manner described by Tanford (1955, 1962). The individual calculated curves for the three regions, plus the fit of the overall calculated curve to the experimental curve, as obtained by this method, are shown in Figure 3.

The results of group assignment supplemented by values from spectrophotometric titration experiments are summarized and compared with data on the total amino acid analysis in Table I. Values for the native and the denatured enzyme are presented in separate columns in order to show the numbers and types of groups that have anomalous pK values in the native enzyme molecule and become normalized only on denaturation. As indicated in Table I, the content of ionizable amino acids (normal plus anomalous), as determined from the titration data, is in satisfactory agreement with the values obtained by direct amino acid analysis (K. D. Schnackerz and E. A. Noltmann, unpublished experiments). Some uncertainty with regard to group assignment remains in the area of carboxyl and imidazole overlap, since back-titration of the acid-denatured enzyme indicates that about 20 groups in this region of the titration curve ionize only on denaturation of the enzyme (*cf.* insert to Figure 3). All of these groups have been counted as histidine residues, since it seems more probable that uncharged imidazole groups, rather than charged carboxyl groups, would be buried in a hydrophobic region; the latter alternative would incur a very unfavorable free-energy change. It is possible that slightly more than 20 groups become normalized on acid denaturation since, due to the continuous manner of the titration, the curves presented in Figure 3 do not represent zero-time values. However, the time dependence of acid denaturation (compare Figure 10) appears to be quite small, so that the uncertainty in the number of buried groups cannot be very substantial.

Estimation of the Electrostatic Factor and of Intrinsic pK Values for the Ionizable Groups. The electrometric titration data for those imidazole groups that titrate within the normal

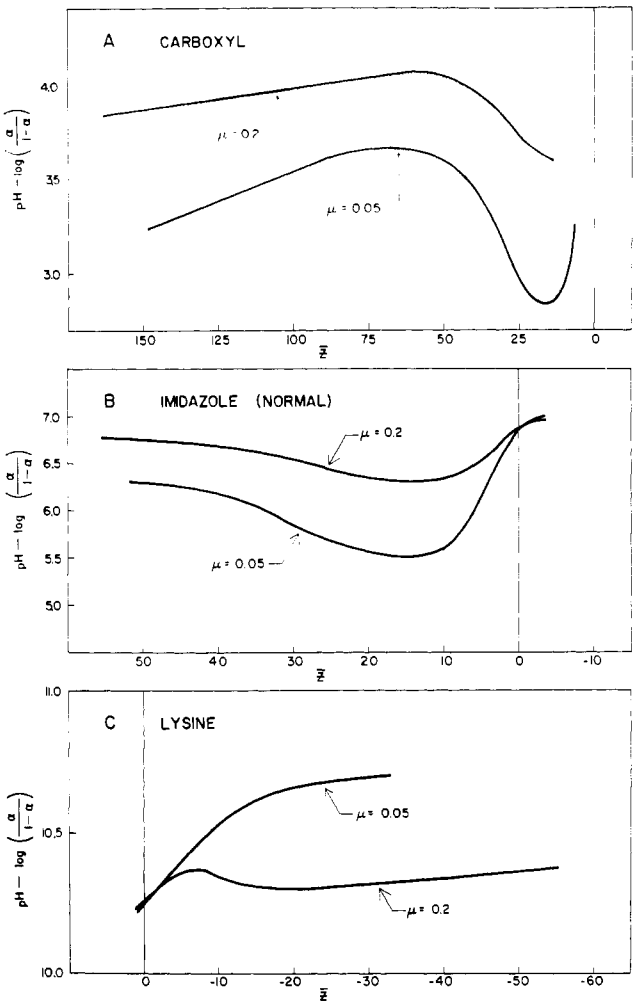

FIGURE 4: Data for the individual titration curves of carboxyl, imidazolyl, and lysyl residues, plotted according to the method of Tanford (1955, 1962). The curves are computed on the basis of the experimental titration curves presented in Figure 3; these were obtained by the continuous titration technique and, therefore, do not yield separate experimental points. In calculating the plots of part C, a value of 82 lysyl residues was used as obtained from the total amino acid analysis (K. D. Schnackerz and E. A. Noltmann, unpublished experiments).

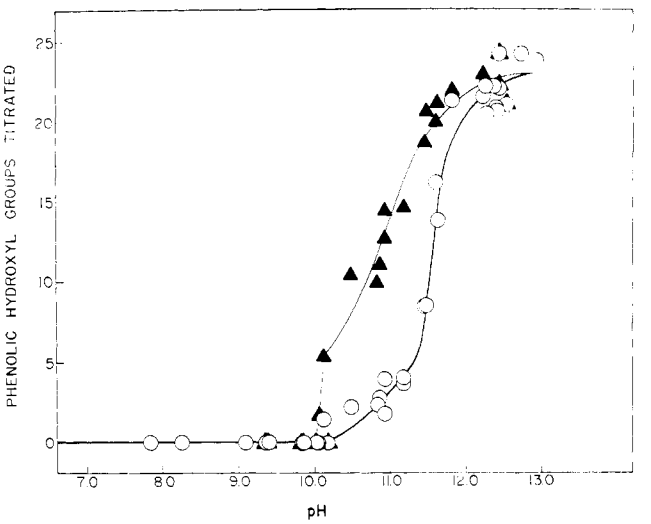

FIGURE 5: Spectrophotometric titration of tyrosine phenolic hydroxyl groups of rabbit muscle phosphoglucose isomerase at 30°. Solvent, 0.2 M KCl (0.2 ionic strength). Enzyme concentrations, 0.10–0.33 mg/ml (0.77–2.50 × 10^{-6} M). Absorbance measurements were made at 300 mμ (and extrapolated to zero time for the zero-time measurements) and converted into the number of phenolic hydroxyl groups as described under Methods. (○) Zero-time values; (▲) equilibrium values, measured after 24-hr incubation at 30°.

pH range (and are assumed to be freely available to the solvent), for lysine groups, and for carboxyl groups, are shown in Figure 4, analyzed according to the method of Tanford (1962). The points corresponding to $\bar{Z}$ values outside the range in which the enzyme can be reversibly titrated (*cf.* Figure 4) are, strictly speaking, not valid according to this method of analysis, since they do not represent a thermodynamic equilibrium. They have been included, however, to demonstrate the marked curvature and change in direction of the slope which takes place at $\bar{Z}$ values corresponding to the pH values where conformational changes begin to occur (compare the spectrophotometric data of Figures 5 and 10). This decrease in slope corresponds to a decrease in the electrostatic factor,[4] which occurs as pH-induced denaturation causes the phosphoglucose isomerase molecule to expand while structural integrity is lost. An increase in the number of available imidazole groups exposed as the solvent gains access to previously screened areas, will also have the effect of decreasing the slope. Finally, dissociation of the enzyme into subunits could be responsible for the phenomenon, since calculation of $\bar{Z}$ values for the lower molecular weight of the subunits would yield a decreased slope compared with that calculated for the molecular weight for the undissociated molecule. Although dissociation has been found to occur in the presence of sodium dodecyl sulfate,[5] it is not known whether it will also be induced by acid or alkaline pH. A definite choice between the possible alternatives can therefore not be made at the present time. It is, however, of interest that the data of Figure 4 are very similar to those reported for the carboxyl groups of bovine serum albumin (Tanford *et al.*, 1955) for which Tanford (1962) also stated that a quantitative explanation is not yet available.

Values for the electrostatic factor, calculated from Figure 4, are shown in Table II. Comparison of experimental values for the electrostatic factor of the native molecule, obtained from the slopes of Figure 4B, with those calculated on the basis of theoretical considerations,[4,6] show that the former are considerably larger than the latter. This discrepancy may be due to the assumption, inherent in the calculation of the electrostatic factor from plots such as those of Figure 4, that all of the groups included in one plot have the same pK. It is

[4] The electrostatic factor is defined as $w = (\epsilon^2/DRkT)[1 - (\kappa R/(1 + \kappa\alpha))]$ (Tanford, 1962).

[5] M. N. Blackburn and E. A. Noltmann, unpublished experiments.

[6] Theoretical values for the electrostatic factor were calculated with a value of 3.65 × 10^{-7} cm for the radius of the phosphoglucose isomerase molecule calculated from the equation $(4/3)\pi R^3 = (M/\mathfrak{N})(\bar{v}_2 + \delta_1 v_1^0)$ (Tanford, 1961), by assuming a value of 0.739 for the partial specific volume (K. D. Schnackerz and E. A. Noltmann, unpublished experiments) and a value of 0.2 for δ_1 (Tanford, 1961).

TABLE II: Theoretical and Experimental Values for the Electrostatic Factor w of Rabbit Muscle Phosphoglucose Isomerase.

Calculation of w	0.2 Ionic Strength	0.05 Ionic Strength
Theoretical[a]	0.022	0.033
From "normal" imidazole ionization[b]	0.066	0.13
From ϵ-amino lysine ionization[c]	0.019	0.023

[a] Calculated as described in the text. [b] Calculated from the initial slopes of Figure 4B, between $\bar{Z} = 0$ and $\bar{Z} = 5$. [c] Calculated from the initial slopes of Figure 4C, between $\bar{Z} = 0$ and $\bar{Z} = -5$.

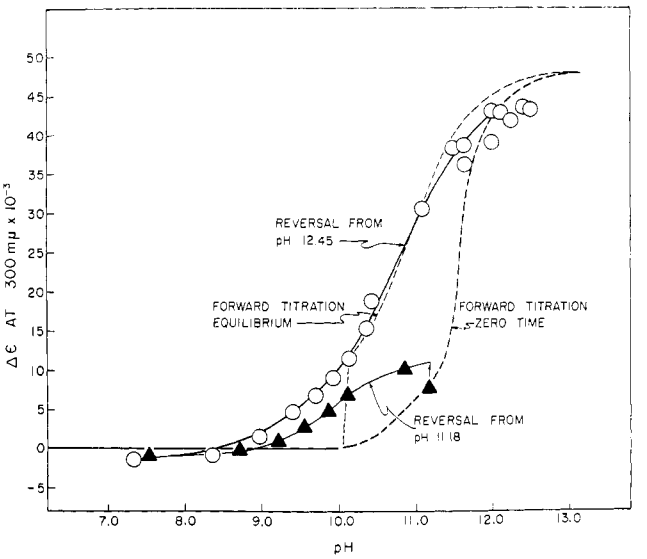

FIGURE 6: Reverse spectrophotometric titration of tyrosine phenolic hydroxyl groups of phosphoglucose isomerase. Conditions were as described in the legend to Figure 5. Reverse titrations were started from pH 12.45 (○) or 11.18 (▲) approximately 3 min after adjustment of the enzyme solution to these pH values. The dotted lines represent zero-time and equilibrium values of the forward titrations shown in Figure 5, superimposed for comparison over the data for the reverse titration.

quite probable, however, that in a large protein molecule, such as phosphoglucose isomerase, local electrostatic interactions will result in a considerable spread of individual pK values. As discussed in some detail by Tanford (1962), this will have the effect of causing the measured electrostatic factor to be higher than the true value. It should be pointed out that the value of the electrostatic factor for the native molecule, calculated from the plot for the lysine groups (Figure 4C), is much closer to the theoretical value. Values for $pK_{intrinsic}$ of carboxyl groups, imidazole groups (normal), and lysine groups, derived from the plots of Figure 4, are given in Table III. Although they are within the normal range for groups of these types in protein molecules, it is probable—in view of the discrepancies noted with respect to the electrostatic factor—that they represent *average* constants rather than apply rigorously to individual groups.

Spectrophotometric Titration of Phenolic Hydroxyl Groups. Spectrophotometric titration curves for the native enzyme are presented in Figure 5 (zero-time and equilibrium values). The maximum ΔA due to ionization of phenolic hydroxyl groups in muscle phosphoglucose isomerase is at 295 mμ. Measurements at this wavelength, when corrected for contributions arising from conformational changes, yield a maximum value of 55,300 for $\Delta\epsilon_{295}$ of tyrosine phenolic hydroxyl groups. If a value of 2330 for $\Delta\epsilon$ per group is assumed (Beaven and Holiday, 1952), a tyrosine content of 23.7 residues may be calculated for rabbit muscle phosphoglucose isomerase, in excellent agreement with the value of 24 obtained by ion-exchange chromatography (K. D. Schnackerz and E. A. Noltmann, unpublished experiments). At a wavelength of 300 mμ, the maximum $\Delta\epsilon$ is equal to 48,500; if a rounded figure of 24 residues is used for the tyrosine content, a value of 2020 for the $\Delta\epsilon_{300}$ per tyrosine residue is obtained. This figure has been used in the conversion of ΔA into residues titrated per phosphoglucose isomerase molecule.

It is apparent (Figure 5) that at zero time no phenolic hydroxyl groups are titrated below pH 10, indicating that in the native enzyme none are sufficiently exposed to the solvent for ionization to take place. The equilibrium curve represents absorbance readings taken after exposure of the enzyme protein for 24 hr at 30° to the pH values indicated, after which time conformational changes at the respective pH values were found to be complete. All of the 24 tyrosine residues are ionized when a pH of about 12.5 is reached, and immediate reverse titration from this pH (Figure 6) produces a curve that is essentially superimposable over the forward titration curve at equilibrium. Titration of the phenolic hydroxyls after denaturation of the enzyme in 8 M urea (Figure 7) produces a titration curve that is also practically identical with the equilibrium curve of Figure 5. The apparent shift by 0.2 pH unit of the curve in urea compared with the equilibrium curve may be

TABLE III: Intrinsic or Estimated pK Values for Ionizable Groups of Rabbit Muscle Phosphoglucose Isomerase.

Ionization	$pK_{intrinsic}$	$pK_{estimated}$
Carboxyl	4.2[a]	
Histidine (imidazole)	6.9[b]	
Lysine (ϵ-amino)	10.2[b]	
Tyrosine (phenolic hydroxyl)		10.8[c]
Cysteine (sulfhydryl)		10.2[d]

[a] Calculated from the midpoint of the curve for carboxyl titration in Figure 3, corrected according to the relationship, $pK_{intrinsic} = pH - 0.868w\bar{Z}$, which holds for $\log \alpha/(1-\alpha) = 0$. [b] From the ordinate intercepts in Figure 4, at $\bar{Z} = 0$. [c] Estimated from the midpoints of the equilibrium curve in Figure 5 and the reverse titration curve in Figure 6; not corrected for electrostatic interaction. [d] Estimated from the midpoint of the titration curve in Figure 9; not corrected for electrostatic interaction.

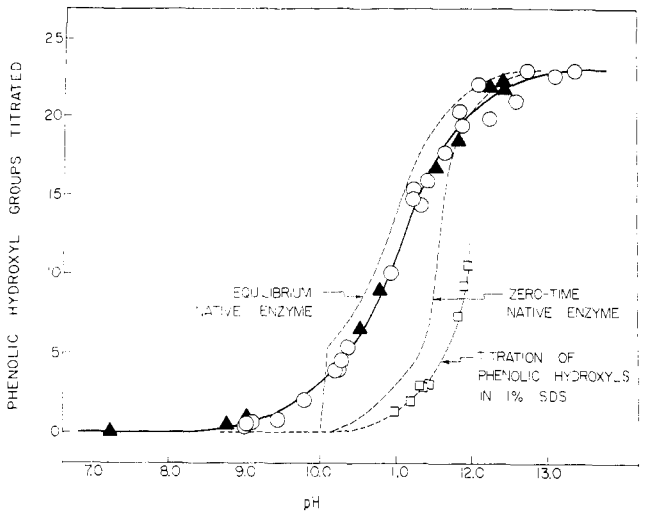

FIGURE 7: Spectrophotometric titration of tyrosine phenolic hydroxyl groups of phosphoglucose isomerase in urea or sodium dodecyl sulfate. Conditions for the titration in the presence of 8 M urea were the same as described in the legend to Figure 5 except that, in addition, the medium was 8 M in urea. (○) Forward titration; (▲) reverse titration, beginning from pH 12.75 downward. Titration in the presence of 1% sodium dodecyl sulfate (SDS) (□) was performed without added KCl to avoid precipitation of the denaturant. Zero-time and equilibrium titration curves, taken from Figure 5, are drawn into the figure for comparison.

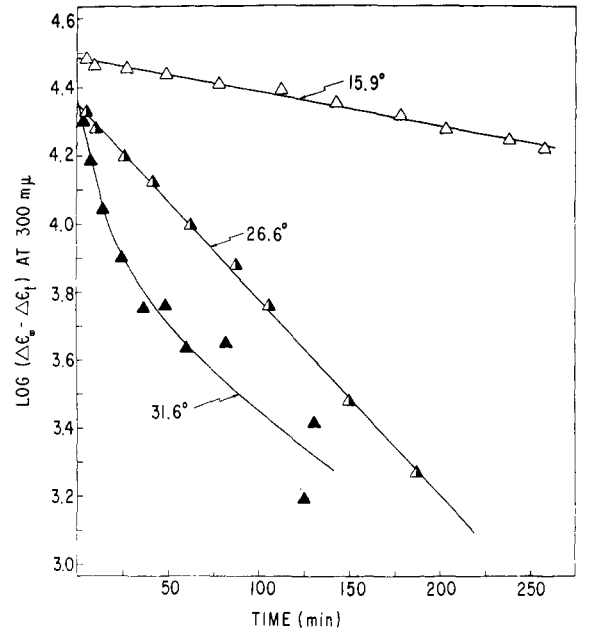

FIGURE 8: Effect of temperature on the titration of tyrosine phenolic hydroxyl groups of phosphoglucose isomerase as a function of time. Absorbance measurements were made at pH 11.1 and 0.2 ionic strength as described under Methods against a reference kept at pH 6.7. Sample temperatures were as given in the figure. $\Delta\epsilon_\infty$ and $\Delta\epsilon$ measured at 300 mμ for the enzyme solution incubated for 24 hr at pH 11.1 and 30°; $\Delta\epsilon_t$ and $\Delta\epsilon$ measured for the enzyme solution incubated at the indicated temperature and pH 11.1 for the time indicated on the abscissa. The lines drawn to points at 15.9 and 26.6° have been calculated as the best fit to the experimental points by least-squares analysis. The line to the points at 31.6° has been drawn as the best visual fit.

an artifact introduced by measurement of the pH in 8 M urea after standardization of the electrodes in aqueous buffer. In the presence of 8 M urea the forward and reverse titration curves coincide, suggesting that throughout the pH range of the titration curve the enzyme is sufficiently denatured to bring all 24 tyrosine residues into equilibrium with the solvent (however, see also the discussion in the accompanying paper (Dyson and Noltmann, 1969)).

The close agreement between the equilibrium curve of Figure 5, the reverse titration curve of Figure 6, and the titration curve in 8 M urea (Figure 7) suggests furthermore that denaturation in time is capable of disorganizing the enzyme molecule to an extent that all phenolic hydroxyl groups are brought into equilibrium with the solvent. However, Figures 5 and 6 yield an apparent pK of 10.8 for the phenolic hydroxyl groups of phosphoglucose isomerase, which is about one unit higher than the expected value (Edsall and Wyman, 1958). It would thus appear that substantial electrostatic interactions between the phenolic hydroxyls and adjacent groups remain after alkaline denaturation and that a considerable degree of structural organization continues to exist.

Examination of the zero-time curve of Figure 6 shows that six or seven phenolic hydroxyl groups are more easily titrated than the remainder, indicating that they may be only partially shielded from the solvent. This conclusion is supported by studies on the time dependence of their ionization at pH 11.1, shown in Figure 8. At temperatures comparable with that at which the titration was performed (30°), *i.e.*, 26.6 and 31.6°, extrapolations to zero time yield seven phenolic hydroxyl groups. Extrapolation to zero time of titration data obtained at 15.9°, on the other hand, yields only three residues immediately ionizable. This difference may be caused by the increased stability of the enzyme at the lower temperature; also, the apparent pK may change with temperature. It will be observed from Figure 8 that at the two lower temperatures the rate of ionization of the phenolic hydroxyl groups obeys apparent first-order kinetics. At 31.6°only the initial part of the slope is of first order, suggesting that at the higher temperature the rate of ionization of tyrosine residues is controlled by two or more factors, such as, *e.g.*, pH in addition to temperature. The observed rate would, therefore, correspond to the sum of two or more rate processes. Calculation of the energy of activation from the increase in rate constant between 15.9 and 26.6° yields a value of 28 kcal/mole for the conformation change that results in the ionization of phenolic hydroxyl groups at pH 11.1 and over the temperature interval indicated.

Spectrophotometric Titration of Sulfhydryl Groups. Attempts at titration of sulfhydryl groups in the native enzyme met with difficulty. Experimental values displayed considerable scatter which, especially at the more alkaline pH values, appears to have been the result of partial oxidation of the sulfhydryl groups; a quantitative interpretation could therefore not be made. Similarly, titration in 8 M urea was unsuccessful, since under the conditions of time, temperature, addition of base aliquots, and mixing, sufficient cyanate was apparently generated in the urea (Stark *et al.*, 1960) to react with the sulfhydryl groups thus as to prevent their ionization. In 1% sodium dodecyl sulfate, however, 12 sulfhydryl groups titrated normally and reversibly (Figure 9), which is in good agreement with their behavior on titration with *p*-mercuribenzoate

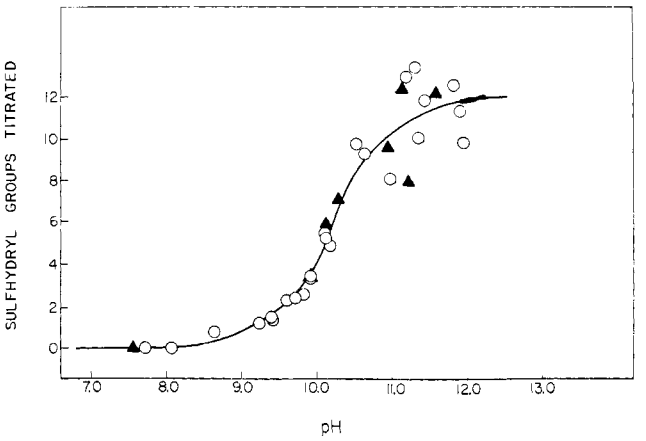

FIGURE 9: Spectrophotometric titration of the sulfhydryl groups of rabbit muscle phosphoglucose isomerase in 1% sodium dodecyl sulfate. Conditions for the titration correspond to those described in the legend to Figure 7 except that the absorbance measurements were made at 245 mμ. (○) Forward titration; (▲) titration reversed from pH 11.9. The total $\Delta\epsilon_{245}$ (extrapolated to zero time and corrected for absorbance contributions from tyrosyl ionization) was found to be 29,750. On the basis of a cysteine content of 12 residues/molecule, this corresponds to a $\Delta\epsilon$ of 2480 per sulfhydryl group and this factor has been used in converting the measured absorbance differences into numbers of sulfhydryls in the figure. This value compares well with a $\Delta\epsilon_{245}$ of 2560 which was obtained by direct measurement of a cysteine solution (0.22 mM) in 1% sodium dodecyl sulfate at neutral pH, against an identical solution at pH 11.9; it is also in reasonable agreement with the value of 2800, found by Donovan (1964) for cysteine in 4 M urea.

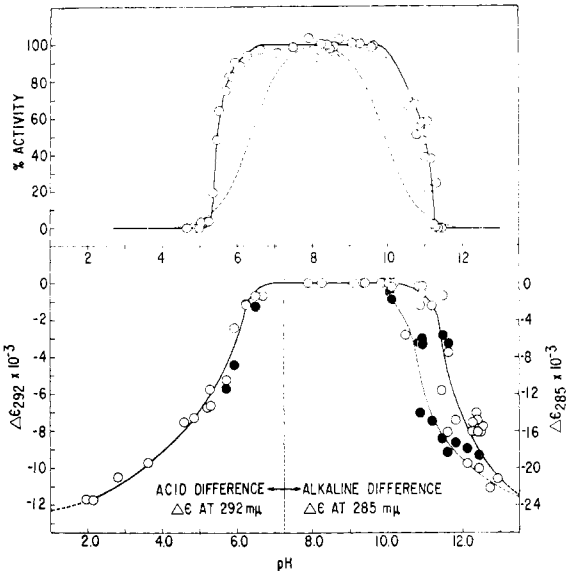

FIGURE 10: Effect of pH on stability and conformation of rabbit muscle phosphoglucose isomerase. For the stability curve (upper figure), enzyme (0.7 mg/ml, 5.3×10^{-6} M) was incubated at 30° in either 0.01 M Tris-acetate or 0.01 M Tris-phosphate buffer, with the ionic strength adjusted to 0.12 by the addition of KCl. After 30 minutes an aliquot was taken and immediately assayed at pH 8.5 and 30° by the pH-Stat assay previously described (Dyson and Noltmann, 1965). The pH values were measured in the enzyme solution at 30°, after the aliquot had been removed for assay. The data are expressed as activity remaining in terms of percent of original activity of the enzyme solution prior to incubation. The dotted curve, included for comparison, represents V_{max} data for the forward reaction as a function of pH, redrawn from a previous paper (Dyson and Noltmann, 1968). Data for the conformational change of the enzyme as a function of pH (lower figure) were obtained at 30° and 0.2 ionic strength. They are represented as differences in the molar absorptivity at either 292 mμ (pH < 7.2) or 285 mμ (pH > 7.2) of a sample at the pH indicated on the abscissa, compared with a reference solution at pH 7.2 (designated by the vertical dotted line). For details, refer to Methods. (○) Zero-time values (pH < 7.2, values were obtained within a 3-min adjustment of pH). (●) Equilibrium values measured after incubation for 24 hr at 30°, and at the indicated pH values.

in the presence of this denaturing agent (Chatterjee and Noltmann, 1967). An apparent average pK of about 10.2 may be estimated from the midpoint of the titration curve shown in Figure 10, which is at the upper limit of accepted pK values for thiol ionization (Benesch and Benesch, 1955).

Effect of pH on Stability and Conformation of Phosphoglucose Isomerase. In order to demonstrate the correlation between conformational changes, which occur within defined pH ranges, and the catalytic functioning of the enzyme, both parameters are shown as a function of pH in the composite Figure 10. The upper part represents the remaining activity (measured at pH 8.5) after exposure of the enzyme at 30° for 30 min to the indicated pH values. For comparison, the catalytic V_{max} curve (from Dyson and Noltmann, 1968) has also been drawn into the same coordinates. In the lower half, the conformational change determined from absorbance measurements at 285 or 292 mμ, as described under Methods, is plotted as a function of pH for data obtained both at zero time and at equilibrium.

The absorbance measurements clearly show a different response of the enzyme to either acid or alkaline denaturation. On the acid side, the conformational change is essentially[7] immediate within the time required for addition of the acid titrant, mixing, and scanning in the spectrophotometer (~2.5 min). In contrast, conformational changes at alkaline pH continued for up to 12 hr (the equilibrium data shown were obtained after 24 hr). It is notable that no conformational change is observed with the spectrophotometric technique between pH 6.3 and 10.0 (lower part of Figure 10) and that these limiting pH values coincide with those at which, under comparable conditions, the enzyme begins to lose its catalytic activity (upper part of Figure 10). Thus, the initiation of conformational changes, as measured by the spectrophotometric method, cannot be brought into agreement with the pK values of 6.75 and 9.3 postulated on the basis of kinetic studies for catalytically critical groups (Dyson and Noltmann, 1968).

Discussion

The data presented here lead to the conclusion that rabbit muscle phosphoglucose isomerase must contain several levels of structural organization which respond differently to various denaturing agents. This is most dramatically exemplified by the finding that denaturation in sodium dodecyl sulfate will expose all of the sulfhydryl groups to the solvent, whereas it

[7] The three equilibrium values for the acid difference spectra, indicated by the solid circles, were actually reached within 30 min and represent the only measurements for which some time dependence was detected on the acid side.

leaves all of the phenolic hydroxyl groups unavailable (*cf.* Figures 7 and 9). A reasonable explanation for this phenomenon would be that sodium dodecyl sulfate, at 1% concentration, is acting only as a dissociating agent, producing individual subunits which have all of their sulfhydryl groups in positions available to the solvent. Such a mechanism for denaturation by sodium dodecyl sulfate would still require that also in the subunits all of the phenolic hydroxyl groups are buried in the nonaccessible interior. An alternative explanation could be that the denaturation induced by sodium dodecyl sulfate is so superficial that all the cysteine side chains are exposed without affecting the screening of the tyrosine phenolic hydroxyl groups. This, however, seems unlikely in view of the demonstration that six or seven tyrosine residues are relatively exposed to the solvent.

A reasonably straightforward interpretation of the conformational changes and their relationship to loss of activity appears to be possible at pH values below 7. Loss of both enzymatic activity and of conformation commences at about pH 6.2 (upper and lower portions, respectively, of Figure 10). The pH dependence of both these parameters is almost parallel down to a pH of about 5.5, indicating that the loss of activity is a result of the pH-induced conformational change. Failure to reverse the acid-induced conformational change, once it has taken place, combined with the lack of an observable time dependence (at 30°) of acid denaturation, suggests that the partially acid-denatured enzyme still retains a portion of its catalytic activity.[8] It would thus appear that the initial acid-induced conformational change is relatively remote from the active center.

Conformational changes accompanying alkali-induced denaturation are more extensive than those caused by acid conditions and rather complex, as indicated by the marked time dependence and stepwise nature of the process. Several different stages of increasingly progressive denaturation may be distinguished which are discussed in more detail in the accompanying paper (Dyson and Noltmann, 1969). It is, however, pertinent at this point to refer to the relationship between conformational changes occurring at alkaline pH and the loss of catalytic activity, and also to the possible participation of tyrosine residues and of histidine residues with anomalous pK values, in maintaining the native structure of the enzyme.

The initial alkali-induced conformational change results in exposure of six or seven tyrosine residues which, according to Figures 5 and 10, amounts to about 12% of the total change that can be achieved by alkaline denaturation (corresponding to the T_B transition described in the accompanying paper (Dyson and Noltmann, 1969)). The full extent of this fraction of the conformational change produces complete loss of catalytic activity. The fact that the activity is only gradually lost, however, suggests that initially the structural alterations must occur relatively remote from the active center which is only affected in the latter stages of this process. The observation of a similar phenomenon during acid denaturation (*vide supra*) may be taken to indicate that the vicinity of the active center is structurally one of the more stable surface regions of the enzyme molecule. It is possible that a minor, *reversible* alteration occurs within the initial alkali-induced conformational change, since the enzyme, after exposure to pH values between 10 and 11.3, still retains partial activity on reversal to pH 8.5, although no catalytic activity is detectable[9] in assays performed above pH 10.1.

Conformational changes leading to loss of enzymatic activity are accompanied on the acid and on the alkaline side by ionization and exposure of about 20 histidine (*cf.* insert to Figure 3) and 24 tyrosine residues, respectively. The apparently concomitant nature of these processes would appear to suggest that the respective residues contribute significantly toward maintaining the native structure of the enzyme. Tyrosine may participate in the formation of hydrophobic bonds by virtue of the hydrophobic nature of its aromatic ring structure. Histidine may contribute to the formation of hydrophobic bonds when the imidazole ring is not ionized.

Both the imidazole nitrogen and the phenolic hydroxyl group may also take part in hydrogen bonding and it is pertinent in this respect that hydrophobic bond formation by a hydrophobic part of an amino acid side chain is considered (Scheraga, 1963) to increase the strength of hydrogen bonding by a polar group of the same side chain. The data of Figure 10 may be taken as supporting the involvement of histidine in hydrogen bonding. Leach and Scheraga (1960a) have pointed out that the pH dependence of the extent of the blue shift, occurring on acid denaturation of a protein, will have the same form as a titration curve of the acceptor group in a hydrogen bond involved in maintaining the native structure of the molecule. The curve in Figure 10 for the pH dependence of $\Delta\epsilon$ at 292 mμ is very similar to a titration curve of an ionizable group with an apparent pK of 5.5. As shown in Figure 3, the midpoint of that portion of the experimental titration curve, which applies to the 20 anomalous histidine residues, occurs also at a pH of about 5.5. It is interesting to note that curves similar to the acid difference curve of our Figure 10 were obtained by Laskowski *et al.* (1960) and by Leach and Scheraga (1960b) for insulin. These authors interpreted their data in favor of a participtation of histidine in hydrogen bonding.

Our data are insufficient to allow identification of those groups presumably engaged in hydrogen bonding with tyrosine and histidine residues. The nearly equal number, however, of imidazole and phenolic hydroxyl groups with anomalous pK values makes it tempting to speculate that they themselves form hydrogen-bonded pairs, which are stabilized by hydrophobic shielding from competition by water molecules. Such a hypothetical arrangement, characterized by juxtaposition of about 20 histidine–tyrosine pairs within the three-dimensional protein structure, would involve about 40 and 80%, respectively, of the total histidine and tyrosine residues present in the enzyme. Although future studies on the three-dimensional structure of phosphoglucose isomerase will have to establish the final validity of these interpretations, the experimental evidence presented here indicates the involvement of tyrosine and histidine in maintaining the native structure of the enzyme.

[8] The alternative possibility, that a pH-dependent equilibrium is established below pH 6.2 between fully active and totally inactive enzyme molecules, seems less likely since such an equilibrium would imply reversibility, which is not observed.

[9] Determinations of enzyme activity were made for this purpose by the pH-Stat assay (Dyson and Noltmann, 1965) under conditions where the added phosphofructokinase was sufficiently stable to permit initial velocity measurements of phosphoglucose isomerase at the alkaline pH (Dyson and Noltmann, 1968).

It may finally be concluded from Figure 10 that the pH values, at which conformational changes begin to occur in either acid or alkaline media, are outside the range that would allow association with the groups having pK values of 6.75 and 9.3, and that have previously been shown to be required in the basic and the conjugate acid form, respectively, for phosphoglucose isomerase to be catalytically active (Dyson and Noltmann, 1968). The original interpretation, therefore, that these two groups are *directly* involved in the catalytic activity of this enzyme, is further supported by the present evidence.

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