Glucose-6-phosphate Dehydrogenase from Brewers' Yeast. The Effects of pH and Temperature on the Steady-State Kinetic Parameters of the Two-Chain Protein Species†

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ABSTRACT: A systematic study has been made of the pH- and temperature-dependency of the steady-state kinetic parameters of the stabilized two-subunit enzyme species of glucose-6-phosphate dehydrogenase, in the absence of superimposed association–dissociation reactions. The $V_{max(app)}$ data obtained in several buffers between pH 5 and 10 and at 18–32 °C lead to the postulate that at least two sets of protonic equilibria may govern the catalysis (one near pH 5.7 at 25 °C and another near pH 9.2); furthermore, two pathways for product formation (i.e., two V_{max}'s) appear to be required to explain the biphasic nature of the log $V_{max(app)}$ vs. pH curves, with V_{max}(basic) > V_{max}(acidic + neutral). Of the several buffers explored, either a uniform degree of interaction or a minimal degree of buffer species interaction could be assessed from the enthalpy changes associated with the derived values for ionization constants attributed to the protonic equilibria in the enzyme–substrates ternary complexes for the case of Tris-acetate–EDTA buffers, at constant ionic strength. With the selection of this buffer at 0.1 ($\Gamma/2$) and at 25 and 32 °C, a self-consistent kinetic mechanism has emerged which allows for the random binding of the two fully ionized substrates to the enzyme via two major pathways, and product formation by both $E{\cdot}A^{-}{\cdot}B^{-}$ and $HE{\cdot}A^{-}{\cdot}B^{-}$. As before (Kuby et al. *Arch. Biochem. Biophys. 165,* 153–178, 1974), a quasi-equilibrium is presumed, with rate-limiting steps (k_{+5} and k_{+5}') at the interconversion of the ternary complexes. Values for the two sets of protonic equilibria defined by this mechanism (viz., pK_κ, pK_{H_2} for the first ionizations, and pK_κ', pK_H' for the second) could then be estimated. From their numerical values (e.g., at 25 °C: $pK_\kappa = 5.7$, $pK_{H_2} = 5.2$; and $pK_\kappa' = 9.1$, $pK_H' = 8.2$) and from the values for $\Delta H°_{ioniz}$ (e.g., $\Delta H°_{pK_\kappa} \simeq 5.1$ kcal/mol; $\Delta H°_{pK_\kappa'} \simeq 11$ kcal/mol), a postulate is presented which attributes these acid dissociation constants to an imidazole and ϵ-amino group, respectively.

D-Glucose-6-phosphate dehydrogenase (Zwischenferment) was first isolated in crystalline form by Noltmann et al. (1961; cf. Kuby et al., 1966) from brewers' yeast as the crystalline NADP-enzyme compound. The *apoenzyme,* i.e., the protein preparation when freed of its coenzyme, $NADP^+$, could also be induced to crystallize (Yue et al., 1967), and interestingly with a geometrically different form than that of the NADP compound (Kuby et al., 1974). The apoenzyme was found to satisfy several criteria of purity (Yue et al., 1967) and a value was assigned for its molecular weight, viz., 102 000. As indicated recently (Kuby et al., 1974), the apoenzyme maintains its integrity without further dissociation into component subunits over a broad range of pH values to ca. 9.8 and in very dilute solutions, as demonstrated by sedimentation equilibrium studies; and this observation will be of some significance in the interpretation of some of the data presented here. Sedimentation equilibrium studies (Yue et al., 1969) in the presence of several disruptive denaturants had permitted the conclusion that the apoenzyme is composed of two noncovalently linked polypeptide chains each of 51 000 molecular weight, with apparently similar, if not identical, physical properties; and since an elemental metal analysis failed to reveal the presence of a metal constituent, glucose-6-phosphate dehydrogenase proved not to be a metalloprotein, unlike a few other dehydrogenases. The NADP-enzyme compound was shown to be the "dimer" of the apoprotein, i.e., to consist of four subunits (Yue et al., 1969; Noltmann and Kuby, 1963). Furthermore, it was also demonstrated (Yue et al., 1969; Noltmann and Kuby, 1963) that the apoenzyme may be titrated with $NADP^+$ and with NADPH (Kuby et al., 1974) to alter successively its hydrodynamic properties. In the absence of interacting ionic species, successful ultracentrifugal titrations were conducted (Yue et al., 1969) and quantitatively interpreted in terms of *a set of* equilibria involving the successive binding of $NADP^+$ to each of the four subunits in the "dimeric" model. Consequently, it had been assumed that there are four binding sites for $NADP^+$ per tetra-subunit protein species, a conclusion which was later confirmed, in principle, by direct measurements of the equilibrium binding of the substrates by the two-subunit apoenzyme (Kuby et al., 1974), stabilized in solution by the controlled use of the $EDTA^{3-}$ ion. Thus, it was found that the $EDTA^{3-}$ ion, if present at significant concentrations (Yue et al., 1969), would completely inhibit the remarkable NADP-induced association phenomenon, an inhibition which was interpreted (Yue et al., 1969) as an effect by the $EDTA^{3-}$ ion on the solvent macromolecular interactions, modifying the dissociation–association equilibria, rather than an effect due to its chelation properties.

Later studies (Kuby et al., 1974) on the ligand-induced macromolecular association to the four-subunit species were extended for $NADP^+$, to include ultracentrifugal titrations at more alkaline pH values, and as before (Yue et al., 1969), significant concentrations of $EDTA^{3-}$ inhibited these mac-

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romolecular association reactions and stabilized the apoenzyme in solution. Although NADPH proved similar to $NADP^+$, in that it induced the formation of the four-subunit species, glucose 6-phosphate^{2-}, by way of contrast, did not (Kuby et al., 1974). In fact, the addition of glucose 6-phosphate^{2-} to NADPH solutions actually inhibited or reversed the association reaction induced by NADPH and, in this respect, the glucose 6-phosphate^{2-} ion acts in a fashion not unlike the $EDTA^{3-}$ ion.

The judicious use of $EDTA^{3-}$ permitted direct steady-state kinetic measurements on the two-chain species alone, which proved to be catalytically active when in the presence of high concentrations of $EDTA^{3-}$ (Kuby et al., 1974). Thus, in the absence of further association–dissociation reactions, the steady-state kinetics of the stabilized (by $EDTA^{3-}$) two-subunit or "monomeric" enzyme species at pH 8.0 (0.10 M glycylglycine) and 25 °C reduced to a simplified mechanism, adequately expressed in terms of a random quasiequilibrium mechanism with a rate-limiting step at the interconversion of the ternary complexes, and with independent binding of the substrates, $NADP^+$ and glucose 6-phosphate^{2-} (Kuby et al., 1974). The product inhibition pattern of NADPH pointed, in addition, to the absence of any kinetically significant concentrations of dead-end complexes with the two-chain species. Equilibrium binding measurements by several techniques (Kuby et al., 1974) confirmed and lent support to these kinetic estimations, in particular that there are two equivalent and independent binding sites for each substrate per 102 000 g of protein unit.

Although it is generally recognized that for bi-substrate reactions, unambiguous information concerning the involvement of protonic equilibria in enzyme catalysis is rarely obtained from the pH dependence of the kinetic parameters, the fact that, in this particular case for the two-subunit catalyst, a rather simplified mechanism seemed to evolve gave indication that pertinent information on the nature of those ionizable groups concerned in the enzymatic reactions might be revealed by a systematic study of the effects of pH and temperature.

The purpose of this communication, therefore, is to present such steady-state kinetic data on the pH and temperature dependence of the glucose-6-phosphate dehydrogenase, when stabilized in solution as the two-subunit catalyst.

Experimental Procedure

Materials

Enzyme Preparations. Glucose-6-phosphate dehydrogenase was isolated by the slightly modified procedure (Kuby and Noltmann, 1966) adapted for large-scale preparations. For the kinetic studies reported here (preparation 20), the enzyme was crystallized five times from aqueous $(NH_4)_2SO_4$ solutions containing $NADP^+$ (Noltmann et al., 1961), until a constant specific activity was reached. The crystalline NADP enzyme was then freed of $NADP^+$ as described (Yue et al., 1967; a ratio of extinction coefficients at 278 to 259 nm of 1.98 was taken as an index of the NADP-free apoprotein); the apoenzyme was crystallized (cf. Yue et al., 1969) for the sixth time and the dissolved crystals (14.72 mg/ml; 540 700 units/ml; see Noltmann et al. (1961) for definition of a unit) were stored at −15 °C in 0.01 M EDTA–0.05 M $(NH_4)_3PO_4$–2.145 M $(NH_4)_2SO_4$, pH 6.8.

Other Materials. $NADP^+$ and D-glucose 6-phosphate (disodium salt) were obtained from Sigma and/or Calbiochem. Solutions of $NADP^+$ were quantitatively analyzed spectrophotometrically and enzymatically with glucose-6-phosphate dehydrogenase and solutions of glucose 6-phosphate analyzed enzymatically also with the use of glucose-6-phosphate dehydrogenase. Crystallized bovine plasma albumin was obtained from Armour.

Other buffers and chemicals were the best available analytical grade commercial products. Redistilled deionized water (degassed by boiling) was employed for the preparations of all solutions, including the several buffers whose compositions are described in the text. The 0.05 M Tris–0.05 M sodium acetate–0.001 M $EDTA^{3-}$ buffers between pH values ca. 5 and 9.5 at 25 and 32 °C were adjusted to give a final ionic strength of 0.10 (when in the reaction mixtures containing glucose 6-phosphate^{2-} and $NADP^{3-}$) by the addition of $NaNO_3$ (ca. 0.044 M), and the pH was adjusted with either standardized NaOH or HNO_3.

Methods

Enzyme Kinetic Measurements. Aliquots of the dissolved, six-times-crystallized apoprotein preparation (No. 20; stored as described above) were diluted to ca. 7.2 μg/ml of enzyme protein in a solution of 5×10^{-2} M EDTA, pH 8.0 at 25 °C, ca. 1 mg/ml of bovine serum albumin, and ca. 0.98×10^{-5} M $NADP^+$, as described in Kuby et al., 1974, under which conditions the enzyme was stable for at least 7 days at 2 °C, with no detectable loss of activity. A further approximately twofold dilution was prepared in 0.05 M EDTA (pH 8) containing 1 mg/ml of albumin, and the reaction was initiated by the addition of 50 μl to 28.0 ml of reaction mixture in a 10-cm (light path) cylindrical cell (whose contents had received a prior temperature equilibration in a thermostated container constructed of Lucite) inserted in the temperature-controlled cuvette housing of a Cary-14 spectrophotometer equipped with a 0–0.1, 0.1–0.2 optical density slide wire. Measurements of initial velocity were thus conducted at a range of ΔOD equivalent to 0.01 full scale, a necessary sensitivity requirement as a result of the comparatively low Michaelis constants encountered for the pyridine nucleotides. Accurately prepared blank cells, without enzyme, proved necessary in order to counterbalance the individual light-absorbancy contributions at this enhanced range of sensitivity. A final concentration of ca. 8×10^{-9} M NADP in the cuvette did not significantly affect the results; nevertheless, this almost insignificant contribution was incorporated within the calculated substrate concentration when the effect of its concentration was explored.

pH Titrations. Over the range of pH 5–7.5, pH titrations of D-glucose 6-phosphate and of $NADP^+$ were carried out with an autotitrator (Radiometer TTT-1-a, pHA 630T, SBR 2C, and SBU1a) operating in the titration mode, at an ionic strength of ca. 0.1 (adjusted with $NaNO_3$) at 25 °C, in a temperature-controlled vessel under a nitrogen barrier, and with standardized NaOH as titrant. Acid dissociation constants (pK_a') were estimated by the derivative method from the recorder charts, and values of 6.08 and 6.13 were measured for the $pK_{a,2}'$ of glucose 6-phosphate and $NADP^+$. These pK_a' values will also be assumed to hold at 32 °C; i.e., their ΔH_{ioniz} are assumed to be less than 300 cal/mol as found in the case of other phosphate esters (Alberty et al., 1951). $pK_{a,2}'$ values were also measured at 0.2 and 0.3 ionic strength and these will be reported later in a report on the effect of various ions and ionic strength on the kinetics of glucose-6-phosphate dehydrogenase.[1]

pH measurements on the buffers and on the reaction mixtures, at each *temperature*, were also made in the thermostated

[1] S. A. Kuby and R. N. Roy, unpublished observations.

titration vessel of the above instrument, or with a Beckman Research pH meter.

Treatment of Data

As demonstrated by Kuby et al. (1974) the most consistent kinetic mechanism, at pH 8.0 and 25 °C, for the two-subunit species of enzyme with two equivalent binding sites for substrate A ($NADP^+$) or B (β-D-glucose 6-phosphate^{2-}) proved to be a quasi-equilibrium random mechanism similar to the one described for the calf brain ATP-creatine transphosphorylase (Jacobs and Kuby, 1970), except that the dead-end complexes, E·B·C or E·A·D could be ignored, and at alkaline pH values an hydroxyl ion catalysis of the product (δ-lactone of 6-phosphogluconate) incorporated. Thus, the steady-state kinetics conformed to first-degree velocity expressions (Wong and Hanes, 1962) for two-substrate reactions and it was presumed that the interconversion of the ternary complexes (E·A·B $\rightleftarrows$ E·C·D) represents the rate-limiting steps; also experimentally it was found that $K_1 \simeq K_4 = \bar{K}_A$; and $K_2 \simeq K_3 = \bar{K}_B$ (see Kuby et al., 1974, for definitions; see Figure 1) resulting in a case of independent binding of A and B. Therefore, the forward initial velocity could be adequately expressed by:

$$V_0^f = V_{max}^f \left[\frac{\bar{K}_A \bar{K}_B}{(A)(B)} + \frac{\bar{K}_B}{(B)} + \frac{\bar{K}_A}{(A)} + 1 \right]^{-1} \quad (1)$$

In the present study over the range of pH values and temperatures studied, under conditions where the two-subunit catalyst prevails (e.g., in the presence of $EDTA^{3-}$ ion), eq 1 appears to adequately express the data. Statistical evaluation of the parameters under each condition of temperature and pH, from either plots of $(V_0^f)^{-1}$ vs. $(S_1)^{-1}$ at several fixed values of (S_2) or from Hultin-type (Hultin, 1967) plots $[(S_1)/(V_0^f)]$ vs. (S_1) at several fixed values of (S_2)] were conducted as described (Kuby et al., 1974) with the aid of a Hewlett-Packard 9820A programmable calculator.

A. Effect of pH. As will be shown later, it was necessary to assume an additional catalytic pathway under basic conditions and the mechanism selected which appears to satisfy all the data is given in Figure 1, which allows for product formation from both $HE{\cdot}A^-{\cdot}B^-$ and $E{\cdot}A^-{\cdot}B^-$ (but *not* from $H_2E{\cdot}A^-{\cdot}B^-$), and where the three ionic forms of the enzyme catalyst are depicted simply as H_2E, HE, and E.

Assuming that, for the forward reactions V_0(overall) = V_0(acidic + neutral) + V_0(basic), it follows that:

$$V_0(\text{acidic + neutral}) = (k_{+5}E_t) \Big/ \left(\left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)} \right] + \frac{K_3}{(B^-)} \left[1 + \frac{(H^+)}{K_\gamma} + \frac{K_\gamma'}{(H^+)} \right] + \frac{K_4}{(A^-)} \left[1 + \frac{(H^+)}{K_\delta} + \frac{K_\delta'}{(H^+)} \right] + \frac{K_1 K_3}{(A^-)(B^-)} \left[1 + \frac{(H^+)}{K_\lambda} + \frac{K_\lambda'}{(H^+)} \right] \right) \quad (2)$$

where $K_1K_3 = K_2K_4$; and

$$V_0(\text{basic}) = (k_{+5}'E_t) \Big/ \left(\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'} \right] + \frac{K_3'}{(B^-)} \left[1 + \frac{(H^+)}{K_\gamma'} + \frac{(H^+)^2}{K_\gamma K_\gamma'} \right] + \frac{K_4'}{(A^-)} \left[1 + \frac{(H^+)}{K_\delta'} + \frac{(H^+)^2}{K_\delta K_\delta'} \right] + \frac{K_1' K_3'}{(A^-)(B^-)} \left[1 + \frac{(H^+)}{K_\lambda'} + \frac{(H^+)^2}{K_\lambda K_\lambda'} \right] \right) \quad (3)$$

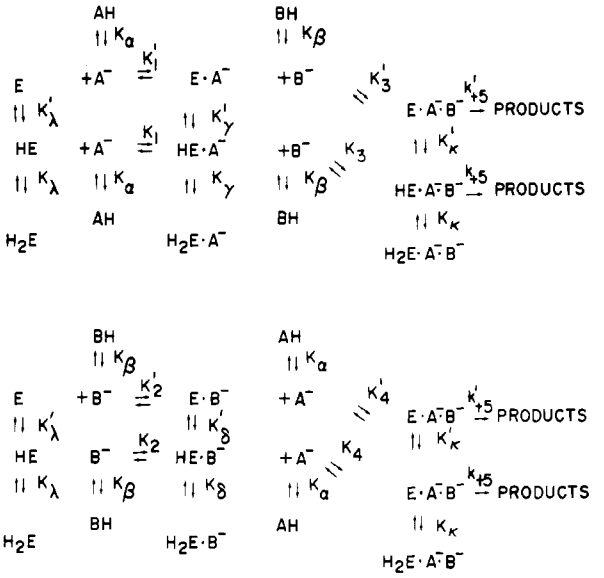

FIGURE 1: Postulated kinetic mechanism for the two-subunit enzyme species. The various ionic, i.e., protonated, states of the enzyme are denoted simply by H_2E, HE, and E.

where $K_1'K_3' = K_2'K_4'$; and also from the rules of symmetry it follows that

$$K_\lambda' K_1 = K_1' K_\gamma'$$
$$K_\lambda' K_2 = K_2' K_\delta'$$
$$K_\delta' K_3' K_4 = K_\gamma' K_4' K_3 \quad (4)$$

As indicated above and as will be justified later; if the following equalities are set

$$\bar{K}_{A^-} = K_1 = K_1' = K_4 = K_4'$$
$$\bar{K}_{B^-} = K_2 = K_2' = K_3 = K_3'$$

it follows from the equalities of eq 4 that $K_\gamma' = K_\delta' = K_\lambda'$, which will be defined as equal to $K_{H'}$; therefore, it is logical to assume also that

$$K_\lambda = K_\delta = K_\gamma = K_{H_2} \quad (5)$$

Thus, both first (K_{H_2}) and second ionizations ($K_{H'}$) of the enzyme's substrate(s) binding sites are assumed to be independent of the binding of either A^- or B^- in the binary complexes, $E{\cdot}A^-$ or $E{\cdot}B^-$, or in the free enzyme (E) but not in the ternary complexes. Therefore

$$V_0(\text{acidic + neutral}) = k_{+5}E_t \Big/ \left(\left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)} \right] + \frac{\bar{K}_{B^-}}{(B^-)} \left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_{H'}}{(H^+)} \right] + \frac{\bar{K}_{A^-}}{(A^-)} \left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_{H'}}{(H^+)} \right] + \frac{\bar{K}_{A^-}\bar{K}_{B^-}}{(A^-)(B^-)} \left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_{H'}}{(H^+)} \right] \right)$$

and

$$V_0(\text{basic}) = k_{+5}'E_t \Big/ \left(\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'} \right] + \frac{\bar{K}_{B^-}}{(B^-)} \left[1 + \frac{(H^+)}{K_{H'}} + \frac{(H^+)^2}{K_{H_2}K_{H'}} \right] + \frac{\bar{K}_{A^-}}{(A^-)} \left[1 + \frac{(H^+)}{K_{H'}} + \frac{(H^+)^2}{K_{H_2}K_{H'}} \right] + \frac{\bar{K}_{A^-}\bar{K}_{B^-}}{(A^-)(B^-)} \left[1 + \frac{(H^+)}{K_{H'}} + \frac{(H^+)^2}{K_{H_2}K_{H'}} \right] \right) \quad (6)$$

The following pH-dependent parameters may be expressed in terms of their pH-independent constants and as a function of the (H^+) ion concentration:

$$V_{max,app}(\text{acidic + neutral}) = V_{*max}(\text{acidic + neutral}) \Big/ \left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right]$$

$$\bar{K}_{B^-,app}(\text{acidic + neutral}) = \bar{K}_{*B^-}\left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_H'}{(H^+)}\right] \Big/ \left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right]$$

and

$$\bar{K}_{A^-,app}(\text{acidic + neutral}) = \bar{K}_{*A^-}\left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_H'}{(H^+)}\right] \Big/ \left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right] \quad (7)$$

and after substitution:

$$V_0(\text{acidic + neutral}) = \frac{V_{max,app}(\text{acidic + neutral})}{1 + \frac{\bar{K}_{B^-,app}}{(B^-)} + \frac{\bar{K}_{A^-,app}}{(A^-)}\left(1 + \frac{\bar{K}_{*B^-}}{(B^-)}\right)} \quad (8)$$

Similarly, for the basic region was defined:

$$V_{max,app}(\text{basic}) = \frac{V_{*max}(\text{acidic + neutral})}{1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}}$$

$$\bar{K}_{B^-,app}(\text{basic}) = \frac{\bar{K}_{*B^-}\left[1 + \frac{(H^+)}{K_H'} + \frac{(H^+)^2}{K_{H_2}K_H'}\right]}{\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}\right]}$$

and

$$\bar{K}_{A^-,app}(\text{basic}) = \frac{\bar{K}_{*B^-}\left[1 + \frac{(H^+)}{K_H'} + \frac{(H^+)^2}{K_{H_2}K_H'}\right]}{\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}\right]} \quad (9)$$

and after substitution:

$$V_0(\text{basic}) = \frac{V_{*max}(\text{basic})}{1 + \frac{\bar{K}_{B^-,app}(\text{basic})}{(B^-)} + \frac{\bar{K}_{A^-,app}(\text{basic})}{(A^-)}\left(1 + \frac{\bar{K}_{*B^-}}{(B^-)}\right)} \quad (10)$$

Finally, in terms of total substrate species (A_t and B_t), i.e., $(A^-) = A_t/(1 + [(H^+)/K_\alpha])$ and $(B^-) = B_t/(1 + [(H^+)/K_\beta])$, and since

$$V_0(\text{overall}) = V_0(\text{acidic + neutral}) + V_0(\text{basic}) \quad (11)$$

$$V_0(\text{overall}) = \left\{V_{max,app}(\text{acidic + neutral}) \Big/ \left(1 + \frac{\bar{K}_{B^-,app}(\text{acidic})}{B_t}\left(1 + \frac{(H^+)}{K_\beta}\right) + \frac{\bar{K}_{A^-,app}(\text{acidic})}{A_t} \times \left(1 + \frac{(H^+)}{K_\alpha}\right)\left[1 + \frac{\bar{K}_{*B^-}}{B_t}\left(1 + \frac{(H^+)}{K_\beta}\right)\right]\right)\right\} + \left\{V_{max,app}(\text{basic}) \Big/ \left(1 + \frac{\bar{K}_{B^-,app}(\text{basic})}{B_t}\left(1 + \frac{(H^+)}{K_\beta}\right) + \frac{\bar{K}_{A^-,app}(\text{basic})}{A_t}\left(1 + \frac{(H^+)}{K_\alpha}\right)\left[1 + \frac{\bar{K}_{*B^-}}{B_t}\left(1 + \frac{(H^+)}{K_\beta}\right)\right]\right)\right\} \quad (12)$$

and if the following are redefined:

$$\bar{K}_{B,app}(\text{acidic}') = \bar{K}_{B^-,app}(\text{acidic})(1 + [(H^+)/K_\beta])$$

$$\bar{K}_{A,app}(\text{acidic}') = K_{A^-,app}(\text{acidic})(1 + [(H^+)/K_\alpha])$$

$$\bar{K}_{*B}' = \bar{K}_{*B^-}(1 + [(H^+)/K_\beta])$$

$$\bar{K}_{B,app}(\text{basic}') = \bar{K}_{B^-,app}(\text{basic})(1 + [(H^+)/K_\beta])$$

$$\bar{K}_{A,app}(\text{basic}') = \bar{K}_{A^-,app}(\text{basic})(1 + [(H^+)/K_\alpha])$$

then it follows:

$$V_0(\text{overall}) = \frac{V_{max,app}(\text{acidic + neutral})}{1 + \frac{\bar{K}_{B,app}(\text{acidic}')}{B_t} + \frac{\bar{K}_{A,app}(\text{acidic}')}{A_t}\left(1 + \frac{\bar{K}_{*B}'}{B_t}\right)} + \frac{V_{max,app}(\text{basic})}{1 + \frac{\bar{K}_{B,app}(\text{acidic}')}{B_t} + \frac{\bar{K}_{A,app}(\text{basic}')}{A_t}\left(1 + \frac{\bar{K}_{*B}'}{B_t}\right)} \quad (13)$$

To evaluate the various pH-independent constants, the following graphical procedures were employed:

I. V_{max} Data. A. From

$$V_{max,app}(\text{acidic + neutral}) = V_{*max}(\text{acidic + neutral}) \Big/ \left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right] \quad (14)$$

in the region between pH 5 to 8, the term K_κ'/H^+ may be neglected in the denominator, and after rearrangement:

$$V_{max,app}(\text{acidic + neutral}) \simeq V_{*max}(\text{acidic + neutral}) - V_{max,app}(\text{acidic + neutral})[(H^+)/K_\kappa] \quad (15)$$

A plot of $V_{max,app}$ vs. $V_{max,app}(H^+)$ yields a value of V_{*max} (acidic + neutral) from the y intercept and $-1/K_\kappa$ from the slope.

B. Similarly, from

$$V_{max,app}(\text{basic}) = V_{*max}(\text{basic}) \Big/ \left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}\right] \quad (16)$$

after neglecting the term $(H^+)^2/K_\kappa K_\kappa'$ in the denominator in the region between pH 8 and 10; and after rearrangement

$$V_{max,app}(\text{basic}) \simeq V_{*max}(\text{basic}) - V_{max,app}(\text{basic})[(H^+)/K_\kappa'] \quad (17)$$

a plot of $V_{max,app}(\text{basic})$ vs. $V_{max,app}(\text{basic})(H^+)$ yields $-1/K_\kappa'$ from the slope and $V_{*max}(\text{basic})$ from the y intercept.

II. K_m Data. A. For the Acidic Region. From

$$\bar{K}_{B,app}(\text{acidic}') = \frac{\bar{K}_{*B^-}\left(1 + \frac{(H^+)}{K_{H_2}} + \frac{K_H'}{(H^+)}\right)\left(1 + \frac{(H^+)}{K_\beta}\right)}{\left(1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right)} \quad (18)$$

in the region between pH 5.4 and 7.4 since it will be shown later that at 25 °C $K_\kappa \simeq 2 \times 10^{-6}$ and $K_\kappa' \simeq 8 \times 10^{-10}$, the term $K_\kappa'/(H^+)$ in the denominator may be neglected compared with $(1 + [(H^+)/K_\kappa])$; similarly it will be shown that $K_H'/(H^+)$ may be neglected compared with $(1 + [(H^+)/K_{H_2}])$ in the numerator. Therefore, eq 18 reduces to

$$\bar{K}_{B,app}(\text{acidic}') \simeq \frac{\bar{K}_{*B^-}(1 + [(H^+)/K_{H_2}])(1 + [(H^+)/K_\beta])}{(1 + [(H^+)/K_\kappa])} \quad (19)$$

which after rearrangement gives

$$\frac{1}{\bar{K}_{B,app(acidic')}}\frac{\left(1+\frac{(H^+)}{K\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa}\right)}=\frac{1}{\bar{K}_{*B^-}}-\frac{1}{\bar{K}_{B,app(acidic')}}\frac{(H^+)}{K_{H_2}}\frac{\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa}\right)} \quad (20)$$

Since K_β (the titration constant of D-glucose 6-phosphate^{2-}) is measured and K_κ estimated from eq 15 (i.e., from $V_{max,app}$(acidic + neutral) data), a plot of the left-hand side vs. $[(H^+)/K_{B,app}(acidic')]\{(1 + [(H^+)/K_\beta])/(1 + [(H^+)/K_\kappa])\}$ yields $1/\bar{K}_{*B^-}$ from the y intercept and $-1/K_{H_2}$ from the slope.

Similarly, from $\bar{K}_{A,app}$(acidic') data:

$$\bar{K}_{A,app}(acidic')=\frac{\bar{K}_{*A^-}\left(1+\frac{(H^+)}{K_{H_2}}+\frac{K_{H'}}{(H^+)}\right)\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa}+\frac{K_\kappa'}{(H^+)}\right)} \quad (21)$$

between pH 5 and 7, $K_\kappa'/(H^+)$ in the denominator and $K_{H'}/(H^+)$ in the numerator may be neglected, giving:

$$\bar{K}_{A,app}(acidic') \simeq \frac{\bar{K}_{*A}\left(1+\frac{(H^+)}{K_{H_2}}\right)\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa}\right)} \quad (22)$$

or after rearrangement

$$\frac{1}{\bar{K}_{A,app(acidic')}}\frac{\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa}\right)}=\frac{1}{\bar{K}_{*A^-}}-\frac{1}{\bar{K}_{A,app(acidic')}}\frac{(H^+)}{K_{H_2}}\frac{\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa}\right)} \quad (23)$$

Since K_α (the titration constant of NADP^{3-}) is measured, and K_κ estimated (eq 15), a plot of the left-hand side vs. $[(H^+)/K_{A,app}(acidic')]\{(1 + [(H^+)/K_\alpha])/(1 + [(H^+)/K_\kappa])\}$ yields $1/\bar{K}_{*A^-}$ from the y intercept, and a second estimate of $1/K_{H_2}$ is obtained from the slope.

B. For the Basic Region. From $\bar{K}_{B,app}$(basic') data since

$$\bar{K}_{B,app}(basic')=\frac{\bar{K}_{*B^-}\left(1+\frac{(H^+)}{K_{H'}}+\frac{(H^+)^2}{K_{H_2}K_{H'}}\right)\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}+\frac{(H^+)^2}{K_\kappa K_\kappa'}\right)} \quad (24)$$

and given the estimates of K_κ and K_κ' that differ by more than three orders of magnitude, at $(H^+) \geq 10^{-8}$ one can safely neglect $(H^+)^2/(K_\kappa K_\kappa')$ in the denominator and, similarly, as will be shown later, the term $(H^+)^2/K_{H_2}K_{H'}$ may be neglected in the numerator, resulting in

$$\bar{K}_{B,app}(basic') \simeq \frac{\bar{K}_{*B^-}\left(1+\frac{(H^+)}{K_{H'}}\right)\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \quad (25)$$

which after rearrangement gives:

$$\frac{1}{\bar{K}_{B,app(basic')}}\frac{\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \simeq \frac{1}{\bar{K}_{*B^-}}-\frac{1}{\bar{K}_{B,app(basic')}}\frac{(H^+)}{K_{H'}}\frac{\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \quad (26)$$

A plot of the left-hand side of eq 26 vs.

$$\frac{(H^+)}{\bar{K}_{B,app(basic')}}\frac{\left(1+\frac{(H^+)}{K_\beta}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)}$$

where K_β is measured and K_κ' estimated from $V_{max,app}$(basic) data (eq 17), yields $1/\bar{K}_{*B^-}$ from the y intercept and $-1/K_{H'}$ from the slope. Similarly, from the $\bar{K}_{A,app}$(basic') data and

$$\bar{K}_{A,app}(basic')=\frac{\bar{K}_{*A^-}\left(1+\frac{(H^+)}{K_{H'}}+\frac{(H^+)^2}{K_{H_2}K_{H'}}\right)\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}+\frac{(H^+)^2}{K_\kappa K_\kappa'}\right)} \quad (27)$$

at pH $\geq$8, both squared (H^+) ion terms in the numerator and denominator may be neglected, resulting in

$$\bar{K}_{A,app}(basic') \simeq \frac{\bar{K}_{*A^-}\left(1+\frac{(H^+)}{K_{H'}}\right)\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \quad (28)$$

and after rearrangement

$$\frac{1}{\bar{K}_{A,app(basic')}}\frac{\left(1+\frac{(H^+)}{K_{\alpha'}}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \simeq \frac{1}{\bar{K}_{*A^-}}-\frac{1}{\bar{K}_{A,app(basic')}}\frac{(H^+)}{K_{H'}}\frac{\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)} \quad (29)$$

A plot of left-hand side vs.

$$\frac{(H^+)}{\bar{K}_{A,app(basic')}}\frac{\left(1+\frac{(H^+)}{K_\alpha}\right)}{\left(1+\frac{(H^+)}{K_\kappa'}\right)}$$

provides a value for $1/\bar{K}_{*A^-}$ from the y intercept and an additional estimate of $-1/K_{H'}$ from the slope.

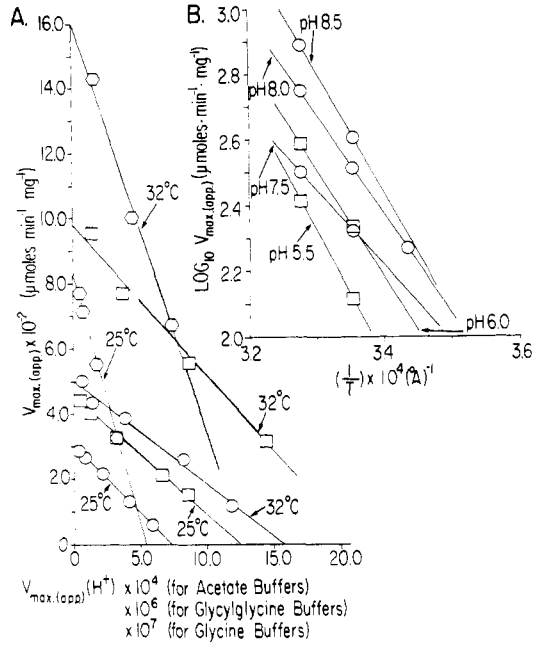

FIGURE 2: (A) Effect of pH on $V_{max,app}$ at several temperatures, i.e., plots of $V_{max,app}$ vs. $V_{max,app}(H^+)$ according to eq 15 and 17 at several pH values and at 25 and 32 °C. (○) Acetic acid (0.1 M) (NaOH)-10^{-3} EDTA; (□) 0.1 M glycylglycine (NaOH)-10^{-3} M EDTA; (□) 0.1 M glycine (NaOH)-10^{-3} M EDTA. (B) Effect of temperature on $V_{max,app}$ at several pH values, i.e., Arrhenius plots of log $V_{max,app}$ vs. $1/T$ at several pH values. (□) Acetate (0.1 M) (NaOH)-10^{-3} M EDTA buffers at pH 5.50 and 6.00; (○) 0.1 M glycylglycine (NaOH)-10^{-3} M EDTA buffers at pH 7.50, 8.00, and 8.50.

For much of the preliminary data, when an estimate for $V_{max,app}$ alone was desired, and a number of conditions explored (buffer species, pH, temperature, etc.), it proved convenient to estimate $V_{max,app}$ by extrapolation of V_0^f data from a simultaneous variation of both substrates, at a fixed ratio of $(NADP^+)/(G\ 6\text{-}P)_0 = 1/4.6$. Thus, if $\beta = (A)_0/(B)_0$, and substitution into eq 1 leads to

$$V_{max}^f/V_0^f = 1 + [\bar{K}_A/(A)_0] + [\bar{K}_B\beta/(A)_0] + [\bar{K}_A\bar{K}_B\beta/(A)_0^2] \quad (30)$$

where it is evident that, in a plot of $1/V_0^f$ vs. $1/(A)_0$, in the $\lim_{1/(A)_0 \to 0} 1/v_0^f \to 1/V_{max}^f$.

$(1/\beta)(1/(B)_0) \to 0$

In most cases and with a judicious selection of the value for β, the extrapolation to $1/V_{max}^f$ appears linear in the region of relatively low values for $1/(A)_0$.

B. Effect of Temperature. I. Thermodynamic parameters were estimated from the intrinsic dissociation or ionization constants, in most cases estimated at 25 and 32 °C. Thus, for $\Delta H°_{ioniz}$, the modified van't Hoff equation, $2.303[pK_1 - pK_2] = (\Delta H°_{ioniz}/R)[(T_2 - T_1)/(T_1 - T_2)]$ was employed; and values for $\Delta F°_{ioniz}$, and $\Delta S°_{ioniz}$ from $\Delta F°_{ioniz} = -RT \ln K_T$ and $\Delta S°_{ioniz} = (\Delta H°_{ioniz} - \Delta F°_{ioniz})/T$, respectively.

II. From $V_{max,app,f}$ data as a function of temperature (in most cases at 18, 25, and 32 °C) in the several buffers at various pH values, the Arrhenius energies of activation (ΔE_{act}) were obtained from the various slopes of log $V_{max,app,f}$ vs. $1/T$ plots, according to $d(\log V_{max,app,f}) = (-\Delta E_{act}/2.303R)d(1/T)$; and after a recalculation of the apparent first-order velocity constant $k_{+5,app}$ (or $k_{+5,app}'$) from $V_{max,app,f}/E_t$ in, units of s^{-1} [i.e., mol of NADPH (mol of catalytic sites)$^{-1}$ s^{-1}]; the thermodynamic parameters for "activation" according to Eyring's absolute rate reaction theory (Stearn, 1949) were calculated. Thus $\Delta H^{\ddagger}$ was obtained from $\Delta H^{\ddagger} = \Delta E_{act} - RT$; $\Delta S^{\ddagger}$, in turn, obtained from $\Delta S^{\ddagger} = (\Delta H/T) + 2.303R[(\log k_{+5,app}) - (\log (RT/Nh))]$; and finally $\Delta F^{\ddagger}$, from $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$.

Results and Discussion

I. The Effects of Temperature on $V_{max,app}$ of the "Monomeric" Enzyme Catalyst in Several Buffer Species at Several pH Values. Arrhenius plots of log $V_{max,app}$ vs. $1/T$ for $V_{max,app}$ values which were estimated over the range of pH 5.5 to 8.5, in acetate and in glycylglycine buffers, are shown in Figure 2B. In Table I, part A, values for the apparent energies of activation (ΔE_{act}) are listed, as well as the calculated values for k_{+5} and the thermodynamic values for activation ($\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$, $\Delta S^{\ddagger}$) computed for 25 °C. It is evident that $k_{+5}^{25\ °C}$ values rise with an increase in pH, then plateau in the region of neutrality, and finally rise again in the alkaline region. On the other hand, ΔE_{act} values appear to decrease with an increase in pH to an apparent minimum near neutrality, followed by a rise in the basic region. An estimated value of ca. 10.6 kcal/mol at pH 7.5 for ΔE_{act} (in 0.1 M glycylglycine–NaOH (10^{-3} M EDTA)) may be compared with an older measurement of 7.1 kcal/mol at pH 7.4 of Glaser and Brown (1955), but obtained under much different ionic environment conditions (viz., 9.5×10^{-2} M Tris-10^{-1} M KCl, 10^{-1} M NH_2OH), and who had measured initial velocities (by hydroxamate formation) rather than maximal velocities. The calculated $\Delta F^{\ddagger}$(25 °C) values appear essentially independent of pH and buffer species, all ca. 14 kcal/mol; however, both the $\Delta H^{\ddagger}$(25 °C) and $\Delta S^{\ddagger}$(25 °C) values appear to be functions of the pH and reach a minimum for $\Delta H^{\ddagger}$(25 °C) of 10 kcal/mol, or change sign to a value of ca. −15 eu for $\Delta S^{\ddagger}$ in the region of neutrality. Thus, either relatively significant conformational changes or isomerizations in the catalyst are reflected in these dramatic effects of pH on the values for $\Delta H^{\ddagger}$(25 °C) or $\Delta S^{\ddagger}$(25 °C), or there are specific interactions of the enzyme catalyst with the buffer species, especially with the protonated glycylglycine species (pK_{a_2}(25 °C) = 8.13), since the pH effects manifest themselves in the pH region below its pK_{a_2}. It is likely that there are some buffer species interactions which, in turn, may result in pH-dependent conformational changes as well as alterations in the pK_a values of these ionizable groups associated with the catalytic process, as will be discussed below.

In Figure 2A, plots are presented according to eq 15 (or 17) in three different buffer systems, to illustrate the effect of pH on $V_{max,app}$ at two temperatures (25 and 32 °C). Firstly, the linear fit of the data to eq 15 (or 17) seems to be quite satisfactory, in all six cases presented, and which cover a broad range of conditions. Accordingly, an evaluation of the pH-independent parameters, V_{max}* and pK_κ (or pK_κ', see Figure 1), may be conducted by means of eq 15 (or 17) and these values are summarized in Table II, part B, together with calculated values for their thermodynamic parameters for ionization, viz., ΔH_{total}^{ioniz}, ΔF_{total}^{ioniz}(25 °C), and ΔS_{total}^{ioniz}(25 °C). The reason for indicating the thermodynamic parameters as "total" values for $\Delta H°$, $\Delta F°$(25 °C), and $\Delta S°$(25 °C) will become apparent shortly. Evidently, there appear to be at *least* two ionizable groups operating in the catalytic process, one in the acidic region of around pH 5.6 and an additional one in the basic region around pH 9.1; whereas, either pK_a may be shifted (but most likely pK_{a_1}, as will be shown below) by interaction with the buffer species, as witnessed by the pK_κ values of ca. 7.6 obtained in the presence of 0.1 M glycylglycine–1 mM EDTA buffers, and the negative enthalpy change of ionization

Table I

A. Effect of Temperature on $V_{max,app}$ at Several pH Values in 0.1 M Acetate–1 mM EDTA (at pH 5.5, 6.0) and 0.1 M Glycylglycine–1 mM EDTA (at pH 7.5, 8.0, 8.5) Buffers

pH	$V_{max,app}$(25 °C) (μmol min^{-1} mg^{-1})	k_{+5}(25 °C) (s)	ΔE_{act} (kcal mol^{-1})	$\Delta H^{\ddagger}$(25 °C) (kcal mol^{-1})	$\Delta F^{\ddagger}$(25 °C) (kcal mol^{-1})	$\Delta S^{\ddagger}$(25 °C) (cal deg^{-1} mol^{-1})
5.50	130	111	17.9	17.3	14.7	+8.8
6.00	215	183	15.0	14.4	14.4	+0.2
7.50	21	179	10.6	10.0	14.4	−14.7
8.00	325	276	13.8	13.3	14.1	−2.9
8.50	403	343	16.7	16.1	14.0	+7.1

B. Effect of pH on $V_{max,app}$ at Several Temperatures

Buffer	T (K)	V_{*max} (mol min^{-1} mg^{-1})	pK_κ or pK_κ'	ΔH_{total}^{ioniz} (kcal mol^{-1})	ΔF_{total}^{ioniz}(25 °C) (kcal mol^{-1})	ΔS_{total}^{ioniz}(25 °C) (eu)
0.1 M acetate	305.2	502	5.5_1			
				7.0	7.7	2.3
10^{-3} M EDTA	298.2	304	5.6_2			
0.1 M GlyGly	305.2	978	7.6_7			
				−6.3	10.3	−55.7
10^{-3} M EDTA	298.2	454	7.5_6			
0.1 M glycine	305.2	1600	9.1_0			
				5.3	12.5	−24.3
10^{-3} M EDTA	298.2	832	9.1_0			

Table II: pH-Independent Kinetic Parameters on Ionization Constants Determined at 25 and 32 °C in 0.1 Ionic Strength Tris-Acetate-EDTA Buffers.[a]

Derived Kinetic Parameter or Ioniz Constant	Defined Reaction	Values: At 25 °C	Values: At 32 °C
pK_α[b]	Titration (ioniz) constant to yield $NADP^{3-}$	6.13[b]	6.13[b]
pK_β[b]	2d ioniz (titration) constant to yield G 6-P^{2-}	6.08[b]	6.08[b]
$\bar{K}_{A^-}$[c]	Intrinsic dissoc constant of enzyme-$NADP^{3-}$ binary or ternary complexes	3.1_4 (± 1.56)[d] 10^{-6} M	5.6_8 (± 2.96)[d] × 10^{-6} M
$\bar{K}_{*B^-}$[c]	Intrinsic dissoc constant of enzyme-G 6-P^{2-} binary or ternary complexes	1.1_5 (± 0.37)[d] × 10^{-5} M	1.4_7 (± 0.76)[d] × 10^{-5} M
pK_{H_2}	1st ioniz constant from enzyme binary complexes or free enzyme	5.2_3	5.4_3
pK_H'	2d ionization constant from enzyme binary complexes or free enzyme	8.1_9	8.1_3
pK_κ	1st ioniz constant from enzyme-substrates ternary complex	5.6_9	5.6_0
pK_κ'	2d ionization constant from enzyme-substrates ternary complex	9.0_8	8.9_0
V_{*max}(acidic + neutral)	$k_{+5}E_t$ or maximal velocity in acidic + neutral range	420 (± 65)[d] μmol/(min mg^{-1})	525 (± 177)[d] μmol/(min mg^{-1})
V_{*max}(basic)	$k_{+5}'E_t$ or maximal velocity in basic range	905 (± 115)[d] μmol/(min mg^{-1})	1810 (± 900)[d] μmol/(min mg^{-1})
k_{+5}	Velocity coefficient for rate-limiting step in acidic + neutral range	357 (± 55)[d] s^{-1}	446 (± 150)[d] s^{-1}
k_{+5}'	Velocity coefficient for rate-limiting step in basic range	769 (± 98)[d] s^{-1}	1539 (± 764)[d] s^{-1}

[a] Buffers: 0.05 M in Tris and acetate; 10^{-3} M in EDTA; ionic strength adjusted with $NaNO_3$ to 0.1. [b] Titration constants to yield fully ionized species of $NADP^{3-}$ and G 6-P^{2-}; values determined for 0.1 ($\Gamma/2$), adjusted with $NaNO_3$. [c] pH-Independent intrinsic dissociation constants for enzyme-fully ionized substrate; thus, in the case of NADP, in its fully ionized state it has a net charge of 3^- (Dixon and Webb, 1958), and for glucose 6-phosphate a net charge of 2^-. [d] Uncertainties in $\bar{K}_{*A^-}$ and $\bar{K}_{*B^-}$ are expressed as standard deviations from the mean, calculated with use of eq 35 and 36, respectively, and for V_{*max}'s with use of eq 32.

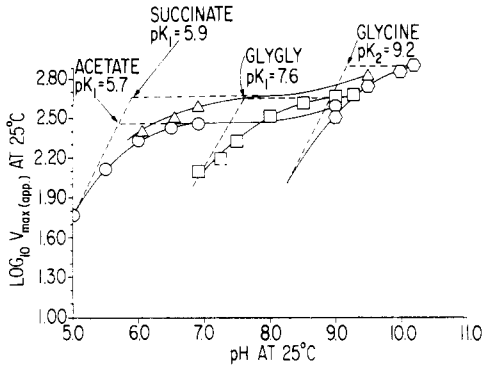

FIGURE 3: Plots of log $V_{max,app}$ vs. pH at 25 °C in several buffers, according to Dixon and Webb (1964). (○) Acetic acid (0.1 M) (NaOH)-10^{-3} M EDTA; (△) 0.1 M succinic acid (NaOH)-10^{-3} M EDTA; (□) 0.1 M glycylglycine (NaOH)-10^{-3} M EDTA; (□) 0.1 M glycine (NaOH)-10^{-3} M EDTA.

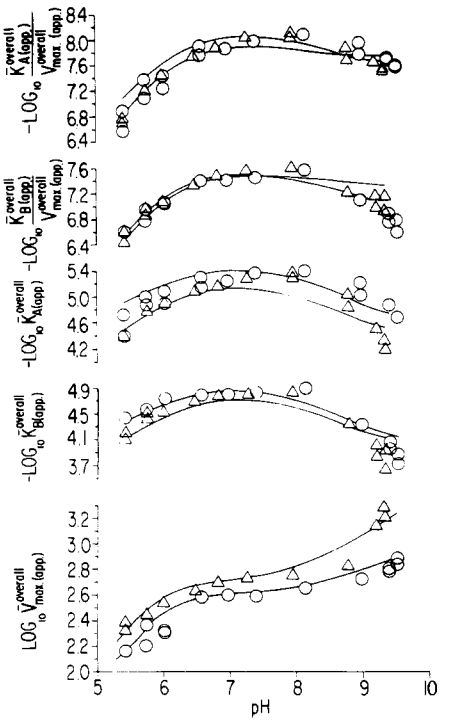

FIGURE 4: Effect of pH on the Michaelis constants and the maximal velocities at 25 and 32 °C, in 0.1 (Γ/2) Tris-acetate-EDTA buffers for the two-chain enzyme species of glucose-6-phosphate dehydrogenase. In all cases: (○) data at 25 °C; (△) data at 32 °C. For further explanation, see Appendix.

(of ca. −6 kcal/mol) associated with this system, together with a relatively large and negative entropy change (or ca. −56 eu). In the other two buffer systems, values for $\Delta H°$ of ionization of ca. 7 and 5 kcal/mol are observed, in 0.1 M acetate-1 mM EDTA and in 0.1 M glycine systems-1 mM EDTA, respectively, with only a modest value for $\Delta S°(25\ °C) = 2.3$ eu for the former, but ca. −24 eu for the latter. Moreover, in the basic region a relatively enormous value for V_{*max} results (in 0.1 M glycine-1 mM EDTA buffers), viz., 1600 μmol min^{-1} mg^{-1} corresponding to a k_{+5}' value of 1360 s^{-1} (or, in the more familiar "turnover" nomenclature, 163 200 mol of NADPH formed per mol of "monomeric" enzyme species per min); this is also associated with a much larger $\Delta F°(25\ °C)$ of ionization of ca. 12.5 kcal/mol compared with the more acidic group ($pK_\kappa \simeq 5.6$) with a $\Delta F(25\ °C)$ of ionization of only ca. 7.7 kcal/mol. Evidently, there are indications, therefore, of a relatively large pH-dependent conformational change (cf. $\Delta S^{ioniz} \simeq -24$ eu) resulting in the exposure of an additional ionizable group in the alkaline region, with associated catalytic function.

In Figure 3 the $V_{max,app}$ data obtained at 25 °C, in four different 0.1 M buffer-1 mM EDTA systems which cover the range from pH 5 to 10, are depicted in terms of Dixon plots (1964). The overall picture which emerges is that there are at least two sets of protonic equilibria governing the catalysis, and that on the basic side an additional pathway with an operable pK near 9 and with a much larger V_{max} than on the acidic side (with an operable pK near 6) would have to be assumed from the biphasic curves of log $V_{max,app}$ vs. pH, and from the second "upward turn" of the data in the basic region. In the acidic region, in acetate or succinate buffers, the shift in $pK_{a,1}$ with buffer species is small, pH 5.7-5.9 (estimated graphically according to Dixon's rules (1964) and shown as dotted lines in Figure 3). Carboxylic anionic buffers would appear to interact less with the system than certain zwitterionic buffers. Thus, a dramatic shift upward of $pK_{a,1}$ to 7.6 is observed in presence of glycylglycine buffers. However, using the data in acetate as a point of reference on the basic side, the $V_{max,app}$ data obtained in glycine buffers would seem to yield approximately a "correct" value for $pK_{a,2}$ of ca. 9.2 at 25 °C.

A comparison was also drawn with Tris buffers (not shown in Figure 3, but see below); thus, at 25 °C in 0.1 M acetate (NaOH)-1 mM EDTA at pH 8.97, a $V_{max,app} = 385$ is measured, and which proved to be almost identical with that measured in 0.1 M Tris (acetate)-1 mM EDTA, at pH 8.97, viz., 364. Therefore, there seems to be an almost insignificant effect of $Tris^+$ compared with Na^+, with $acetate^-$ as the counterion, and similar data were obtained over the pH range of ca. 5.5-9.0. An "ideal" buffer system, with minimum interaction, or at least a constant degree of interaction, would appear to be that of a Tris-acetate-EDTA system, and under controlled ionic strength conditions to yield meaningful and interpretable data on the catalytically operable protonic equilibria. These data on the pH effects, both on the $K_{m,app}$'s and on $V_{max,app}$'s, will be presented next, following a brief but semiquantitative estimation of the degree of buffer species interaction found here. Thus, consider the data given in Table I, part B. If it may be assumed that the $\Delta H°$ of ionization measured in 0.1 M acetate-1 mM EDTA buffers represents an ionizable group (ΔH^a) in the enzyme protein with a $pK_\kappa \simeq 5.6$, and that a value of ΔH_{total}^{ioniz} in 0.1 M glycylglycine-1 mM EDTA represents an overall (or total) heat of ionization, including that of the interacting buffer (ΔH^b). Thus, $\Delta H_{total}^{ioniz} = \Delta H^a$(ioniz of enzyme group) − ΔH^b(ioniz of buffer); or $-6.3 \times 10^3 = 7.0 \times 10^3 - \Delta H^b$; $\Delta H^b = (7.0 + 6.3)10^3 = 13.3$ kcal/mol. Murphy and Martell (1957) measured a $pK_{a,2} = 8.01$ for glycylglycine at 0.09 ionic strength (with KCl) and 30 °C, and a $\Delta H°$ of ionization for $pK_{a,2} \simeq 12.0$ kcal/mol; a value not too dissimilar to that calculated here.

II. The Effect of pH on $K_{m,app}$ for Each Substrate and on $V_{max,app}$ for the Two-Chain Enzyme Catalyst at 0.1 Ionic Strength (Tris-Acetate-EDTA) at 32 and 25 °C. Figure 4 summarizes all the data obtained for $V_{max,app}$ and $K_{m,app}$ for $NADP^+$ and glucose 6-phosphate at a fixed ionic strength of 0.1 in 0.05 M Tris-0.05 M acetate-1 mM EDTA buffers, at both 25 and 32 °C.

The lowest set of plots is given in terms of log $V_{max,app}$ vs. pH, and it is evident that the $V_{max,app}$ values rise smoothly to a plateau in the region of ca. pH 7 and then continue to further increase in the basic region in a concave-upward fashion. As

indicated above, under the "Treatment of Data", it is necessary to assume a second pathway for product formation in the basic region (see Figure 1), and the minimum assumptions necessary to account for these data provide for product formation from the ternary complexes, $EH{\cdot}A^-B^-$ as well as $E{\cdot}A^-B^-$, but not from $EH_2{\cdot}A^-B^-$ whose concentration would become negligible above pH 7. Furthermore, it is proposed that the enzyme may bind either ionized substrate, in a random fashion, via two major pathways. By making the additional assumption that the first ionization constant K_{H_2} in the free enzyme's active center was not altered on binding either of the ionized substrates to yield the respective binary complexes, and similarly with the second ionization constant K_H', it proved possible to evaluate the two ionizations in the ternary complexes (K_κ and K_κ') which are altered on binding of the second substrate. Evaluations of K_κ and K_κ' were made in the acidic and basic regions of the data, respectively, by the graphical procedures described in the text (see eq 15 and 17), which also yielded values for the pH-independent maximal velocities. The final data were then "smoothed" by minor adjustments with the aid of the "theoretical" expressions:

$$V_{max,app}(\text{overall}) = V_{max,app}(\text{acidic + neutral}) + V_{max,app}(\text{basic}) \quad (31)$$

$$V_{max,app}(\text{overall}) = \frac{V_{*max}(\text{acidic + neutral})}{1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}} + \frac{V_{*max}(\text{basic})}{1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}} \quad (32)$$

(See above (under "Treatment of Data", eq 13–17)). With the use of those values for pK_κ and pK_κ' and for V_{*max}(acidic + neutral) and V_{*max}(basic) listed in Table II, as may be seen, the fit of the data to the above theoretical expressions (solid lines in log $V_{max,app}$(overall) vs. pH curves of Figure 4) at both 25 and 32 °C are quite satisfactory, lending credence to the overall treatment of the maximal velocity data. Similarly, by defining

$$\bar{K}_{B,app}(\text{overall}) = \bar{K}_{B,app}(\text{acidic + neutral}) + \bar{K}_{B,app}(\text{basic}) \quad (33)$$

$$\bar{K}_{A,app}(\text{overall}) = \bar{K}_{A,app}(\text{acidic + neutral}) + \bar{K}_{A,app}(\text{basic}) \quad (34)$$

and after substitution from eq 18, 21, 24, and 27, the following expressions for the H^+ ion dependency of $\bar{K}_{B,app}$ and $\bar{K}_{A,app}$ result:

$$\bar{K}_{B,app}(\text{overall}) = (\bar{K}_{*B^-})\left(1 + \frac{(H^+)}{K_\beta}\right)\left\{\frac{\left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_H'}{(H^+)}\right]}{\left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right]} + \frac{\left[1 + \frac{(H^+)}{K_H'} + \frac{(H^+)^2}{K_{H_2}K_H'}\right]}{\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}\right]}\right\} \quad (35)$$

$$\bar{K}_{A,app}(\text{overall}) = (\bar{K}_{*A^-})\left(1 + \frac{(H^+)}{K_\alpha}\right)\left\{\frac{\left[1 + \frac{(H^+)}{K_{H_2}} + \frac{K_H'}{(H^+)}\right]}{\left[1 + \frac{(H^+)}{K_\kappa} + \frac{K_\kappa'}{(H^+)}\right]} + \frac{\left[1 + \frac{(H^+)}{K_H'} + \frac{(H^+)^2}{K_{H_2}K_H'}\right]}{\left[1 + \frac{(H^+)}{K_\kappa'} + \frac{(H^+)^2}{K_\kappa K_\kappa'}\right]}\right\} \quad (36)$$

It is, of course, important to note that, whereas two pH-independent maximal velocities, V_{*max}(acidic + neutral) and V_{*max}(basic), are required as a minimum assumption to account for the data, only one value is required for each of the pH-independent intrinsic dissociation constants for the ionized substrates, i.e., $\bar{K}_{*A^-}$ or $\bar{K}_{*B^-}$, throughout the entire pH range.

Nevertheless, the *apparent values* for the Michaelis constants are very strongly dependent on pH. Thus, e.g., at 25 °C, $\bar{K}_{B,app}$(overall) decreases from 3.8×10^{-5} at pH 5.41 to ca. 1.3×10^{-5} at pH 8.13 and then rises almost tenfold to ca. 14×10^{-5} at pH 9.16; similarly, $\bar{K}_{A,app}$(overall) decreases from ca. 20×10^{-6} at pH 5.41 to 4.2×10^{-6} at pH 8.13, and then rises fivefold to ca. 22×10^{-6} at pH 9.16. The combination of a tenfold rise in $\bar{K}_{B,app}$ and a fivefold rise in $\bar{K}_{A,app}$ at pH 9.5 vs. 8.1 would result in an extremely sharp *decrease* in the "initial velocity", with an increase in pH above pH 9, if fixed concentrations of both substrates were employed to explore the "variation in activity with pH" as, for example, in the early results of Glaser and Brown (1955), who found an apparently sharp pH optimum of ca. pH 8.5 at 25 °C and a steep decline above pH 9.5. Moreover, it has been a common observation, especially amongst those who have employed glucose-6-phosphate dehydrogenase in "coupled enzyme assays", that above pH 9.5–10, the reaction rate appears to decrease dramatically. On the other hand, as described earlier and as will be noted below, V_{max}(overall) data at 25 °C in fact rise from ca. 144 μmol/min^{-1} mg^{-1} at pH 5.41 to ca. 442 at pH 8.13 and then increase still further to ca. 697 at pH 9.52.

The middle two plots of log $K_{B,app}$ and of log $\bar{K}_{A,app}$ vs. pH at 25 and 32 °C in Figure 4 were drawn with the aid of the above "theoretical" curves described by eq 35 and 36 (the solid lines). Calculations were greatly facilitated with the use of a Hewlett-Packard 9820 programmable calculator and, as may be seen, these fits to the experimental data also appear satisfactory. A statistical evaluation of the two pH-independent $\bar{K}_{*A^-}$ and $\bar{K}_{*B^-}$ parameters (i.e., the deviations from the theoretical curve) was conducted and their standard deviations are given in Table II. The value of $\bar{K}_{*A^-}$ at 25 °C of 3.14×10^{-4} may be compared with the intrinsic dissociation constant of $2.65 (\pm 0.54) \times 10^{-6}$ (estimated directly (Kuby et al., 1974) by difference spectroscopy, but under somewhat different conditions, viz., $(\Gamma/2) \simeq 0.2$ at 29 °C and pH 7.5, and 10 mM EDTA).

Finally, as an overall test of fitness to the theory presented, the upper plots in Figure 4 were constructed, viz., that of $-\log(\bar{K}_{A,app}/V_{max,app})$ vs. pH, or of $-\log(\bar{K}_{B,app}/V_{max,app})$ vs. pH, which then incorporate all of the derived constants, and which would tend to exaggerate any unusual discrepancies from the proposed mechanism. No serious departures from the solid lines (theoretical curves) are manifested, and Table II provides a summary of all of the derived kinetic parameters, together with the estimated uncertainties in the pH-independent parameters.

Table III: Effect of Temperature on the pH-Independent Ionization Constants and Intrinsic Dissociation Constants in 0.1 Ionic Strength Tris–Acetate–EDTA Buffers.

pH-Independent or Intrinsic Dissoc Constant	T (K)	Value	ΔH_{total}^{ioniz} or $\Delta H°$ (kcal/mol)	ΔF_{total}^{ioniz}(25 °C) or $\Delta F°$(25 °C) (kcal/mol)	ΔS_{total}^{ioniz}(25 °C) or $\Delta S°$(25 °C) (cal/(deg mol^{-1})
pK_{H_2}	305.2	5.4_3	−11.8	7.1	−63.6
	298.2	5.2_3			
pK_H'	305.2	8.1_3	4.0	11.2	−24.1
	298.2	8.1_9			
pK_κ	305.2	5.6_0	5.1	7.8	−9.1
	298.2	5.6_9			
pK_κ'	305.2	8.9_0	10.8	12.4	−5.4
	298.2	9.0_8			
$p\bar{K}_{*B^-}$	305.2	4.8_3	6.3	6.7	−1.4
	298.2	4.9_4			
$p\bar{K}_{*A^-}$	305.2	5.2_5	15.3	7.5	26.1
	298.2	5.5_0			

Table IV: Effect of Temperature on the pH-Independent Maximal Velocities in 0.1 Ionic Strength Tris–Acetate–EDTA Buffers.

pH-Independent Maximal Velocity	T (K)	V_{*max} (μmol/(min mg^{-1}))	k_{+5} or k_{+5}' (s^{-1})	ΔE_{act} (kcal/mol)	$\Delta H^{\ddagger}$(25 °C) (kcal/mol)	$\Delta F^{\ddagger}$(25 °C) (kcal/mol)	$\Delta S^{\ddagger}$(25 °C) (cal/(deg mol^{-1})
V_{*max}(acidic + neutral)	298.2	420	357				
				5.8	5.2	14.0	−29.5
	305.2	525	446				
V_{*max}(basic)	298.2	905	769				
				17.9	17.3	13.5	12.7
	305.2	1810	1539				

In Table III, the effect of temperature on the pH-independent ionization constants and intrinsic dissociation constants is described in terms of the calculated thermodynamic parameters. Thus, from the pK_κ values of 5.6 and 5.7, a $\Delta H°$ of ca. 5 kcal/mol (Table III) may be estimated for the enthalpy change associated with the first ionization reaction in the enzyme-ternary compound (i.e., to HE·A$^-$·B$^-$), and which may be compared with a $\Delta H°$ of ca. 11 kcal/mol estimated from the pK_κ' values (of 8.9 and 9.1, at 32 and 25 °C, respectively) as ascribed to the second ionization reaction in the enzyme ternary compound (i.e., to E·A$^-$·B$^-$). A $pK_{a,1}$ of 5.7 at 25 °C with a $\Delta H°_{ioniz}$ of ca. 5.1 kcal/mol may also be compared with a likely range of 5.6–7.0 for imidazolium groups in proteins as given by Cohn and Edsall (1943) and with a range of associated $\Delta H°$ values of 6900–7500 cal/mol. Similarly, a $pK_{a,2}$ of 9.1 at 25 °C with $\Delta H°_{ioniz}$ of 10.8 kcal/mol may be compared with the estimates of Cohn and Edsall (1943) for an ϵ-ammonium group of a lysyl residue, viz., a pK_a range of 9.4–10.6 with a $\Delta H°$ range of 10–12 kcal/mol.

Interestingly, pK_κ decreases at 25 °C from 5.7 in the enzyme ternary compound to 5.2 in the case of pK_{H_2} for the enzyme binary compound or the free enzyme; and similarly, pK_κ' decreases from 9.1 at 25 °C to 8.2 in pK_H'. Moreover, the associated values for ΔH_{total}^{ioniz} become −12 and 4 kcal/mol in the case of pK_{H_2} and pK_H', respectively (cf. Table III).

If one assumes that the shifts in pK_a's are due to interaction with an adjacent functional group in either the enzyme protein or in the enzyme–substrate binary compound and that this interaction is diminished on binding the second substrate, one can estimate, as before, the degree of interaction with this group from the enthalpy changes. Thus, in the basic range

$$\Delta H_{total}^{ioniz} = \Delta H^a(\text{ioniz of enzyme group}) - \Delta H^b(\text{ioniz of interacting group in protein})$$

and after substitution of ΔH_{ioniz}^a = 10.8 kcal/mol derived from the pK_κ' data, and 4.0 kcal/mol = ΔH_{total}^{ioniz} from the pK_H' data, i.e., $4.0 \times 10^3 = 10.8 \times 10^3 - \Delta H^b$; $\Delta H^b = (10.8 - 4.0)10^3 = 6.8$ kcal/mol for the enthalpy of ionization at 25 °C of this hypothetical interacting group in the enzyme protein. This value for $\Delta H_{ioniz}°$ may be compared with a value of $\Delta H° = 6.9$ kcal/mol obtained by Benesch and Benesch (1955) for the ionization of the –SH group in thioglycolic acid. Moreover, Kuby et al. (1974) had found that the rates of action with DTNB and a *single* thiol group per subunit, as revealed by its rate of inactivation of the enzyme, could be *reduced* in the presence of *either* substrate, $NADP^+$ or D-glucose 6-phosphate^{2-}, an observation which implicates a thiol group in the active conformation of the enzyme. If one makes a similar calculation for the degree of interaction of this group in the acidic range, i.e., from ΔH data derived from values of pK_{H_2} and pK_κ, then $\Delta H^b = (5.1 + 11.8)10^3 = 16.9$ kcal/mol, or almost twice the heat or ionization for presumably the same group as found in the alkaline region. Part of this larger heat of ionization may be logically attributed to other coupled interacting reactions which may be underlying this set of protonic equilibria.

Finally, from the intrinsic dissociation constants of the ionized substrate and the enzyme, $p\bar{K}_{*B^-}$ and $p\bar{K}_{*A^-}$, $\Delta H°$ values of 6.3 and 15 kcal/mol, respectively, are obtained (Table III), with only a minimal value for $\Delta S°$(25 °C) in the case of $p\bar{K}_{*B^-}$ (−1.4 eu), but with a much larger and positive entropy value of 26 eu associated with $p\bar{K}_{*A^-}$; the values for $\Delta F°$(25 °C) in both cases remain fairly constant at 6.7 and 7.5 kcal/mol for $p\bar{K}_{*B^-}$ and $p\bar{K}_{*A^-}$, respectively. These data of Table III will be discussed further in some detail below.

In Table IV, the derived values for the pH-independent maximal velocities and their associated thermodynamic values for activation are listed. Thus, as indicated, the velocity data

require the incorporation in the mechanism of two pH-independent maximal velocities, one for the acidic and neutral region, V_{max}(acidic + neutral) (with a value, e.g., of ca. 525 at 32 °C), and a second maximal velocity in the basic region, V_{*max}(basic) (with a value more than three times its counterpart in the acidic region, e.g., 1810 μmol min^{-1} mg^{-1} at 32 °C). Derived values for k_{+5} and k_{+5}' (see Figure 1) are also listed in Table IV together with their apparent energies of activation ΔE_{act}, and values for $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$, $\Delta S^{\ddagger}$ at 25 °C. Interestingly, ΔE_{act} differs for the two sets of "turnover numbers", with only ca. 5.8 kcal/mol in the acidic and neutral region compared with almost 18 kcal/mol (or ca. 17 kcal/mol for $\Delta H^{\ddagger}$) in the basic region; again $\Delta F^{\ddagger}$(25 °C) remains fairly constant in both sets, viz., ca. 14 kcal/mol; but $\Delta S^{\ddagger}$(25 °C) is almost −30 eu for the acidic region compared with +12.7 for the basic region, with implications of larger entropic influences in the acidic and neutral region, but with a greater potential-energy barrier in the basic region. If one accepts Pauling's estimates of hydrogen-bond energies (Pauling, 1948) viz., ca. 1–8 kcal/mol, then from the energetics of the system as described by $\Delta H^{\ddagger}$(25 °C) $\simeq$ 5 kcal/mol on the acidic and neutral side, it would permit at least one hydrogen bond to be broken in the "transition state", and possibly three on the basic side.

Overall Discussion

The possibility of inhibiting association-dissociation reactions of the enzyme protein and stabilizing the enzymatically active species in a single form, viz., that of the two subunit catalyst (Kuby et al., 1974; Yue et al., 1969), has presented a unique situation for the exploration of its kinetic mechanism. Over the broad range of conditions studied here—with respect to pH, temperature, and buffer species—the two-subunit enzyme species seemed to follow the same simplified kinetic mechanism previously described (Kuby et al., 1974). With a few logically arrived at assumptions and justified by the self-consistency of the final analysis (as given in Figure 4), the complex nature of the pH dependency could be quantitatively described in terms of two sets of catalytically operable protonic equilibria, after some of the effects of interacting buffer species (see e.g., Figure 2A) could be assessed. Thus, it is assumed that the enzyme may bind the fully ionized species of substrate (G 6-P^{2-} or NADP^{3-}) in a random fashion via two major pathways and that all species of enzyme-substrates ternary complexes, except $H_2E{\cdot}A^-{\cdot}B^-$, break down to products. (Further, the steady-state concentrations of H_2E, $H_2E{\cdot}A^-$, $H_2E{\cdot}B^-$, and $H_2E{\cdot}A^-{\cdot}B^-$ will reduce to insignificant values above neutral pH's, if the estimates for the pH-independent kinetic parameters are only roughly correct, see below and Table II.) This leads to a mechanism with *two* pH-independent maximal velocities operating over two pH ranges, viz., V_{*max}(acidic + neutral) and V_{*max}(basic), and which accounts adequately for the biphasic nature of $V_{max,app}$(overall) vs. pH plots obtained in the presence of a system of minimally (or of fixed degree) interacting buffer species, i.e., Tris–acetate–EDTA (see Figure 4, lowest set of plots, which are described by eq 31 and 32). But, on the other hand, after correction for the ionization of the substrates (by means of the measured values of pK_α and pK_β, i.e., to yield NADP^{3-} and G 6-P^{2-}, respectively), it was found that only a *single* pH-independent intrinsic dissociation constant for each substrate, $\bar{K}_{*A}$- and $\bar{K}_{*B}$-, was necessary to account for the pH dependency of either $\bar{K}_{A,app}$(overall) or $\bar{K}_{B,app}$(overall) in the Tris–acetate–EDTA system of buffers (see Figure 4, middle set of two plots, which in turn are described by eq 33–36). Further, the protonic equilibria operating

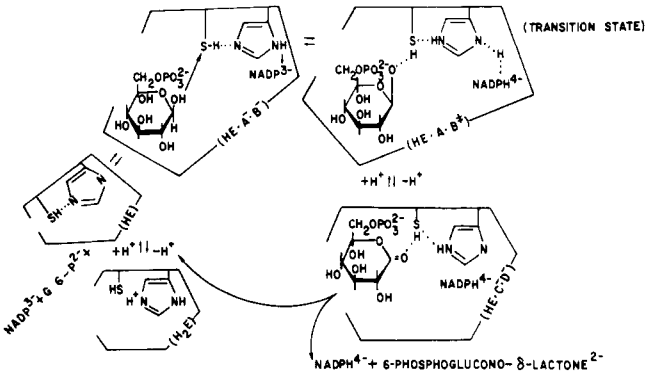

FIGURE 5: Postulated kinetic mechanism of dehydrogenation by D-glucose-6-phosphate dehydrogenase in the acidic and neutral pH range.

in the enzyme-ternary complexes are defined simply by K_κ (for $K_{a,1}$) and K_κ' (for $K_{a,2}$); and these same ionizable groups, with their associated ionization constants now altered to K_{H_2} and K_H' are postulated to be present in the enzyme-binary complexes or in the uncombined enzyme.

Assigned values for the two sets of catalytically operable protonic equilibria, viz., for the pK_κ–pK_{H_2} set and for the pK_κ'–pK_H' set, are given in Table II at 25 and 32 °C; and the thermodynamic parameters associated with these two sets of ionizations are summarized in Table III. From the estimated value of pK_κ(25 °C) $\simeq$ 5.7 with a $\Delta H^\circ_{ioniz} \simeq$ 5 kcal/mol; the first set is ascribed to a proton dissociation from an imidazolium group (cf. Cohn and Edsall, 1943) as inferred above in the Results section; also, from the value of p$K_\kappa' \simeq$ 9.1 with a $\Delta H_{ioniz} \simeq$ 11 kcal/mol, the second set is deduced to be the result of a proton dissociation from an ϵ-ammonium group of a lysyl residue (again, cf. Cohn and Edsall, 1943).

A single-SH group per subunit has been proposed (Kuby et al., 1974) as involved in the "transition" state or in the active conformation, since *either* substrate will protect against inactivation by 5,5′-dithiobis(2-nitrobenzoic acid). Therefore, it is plausible to suggest that the thiol group lies in close proximity to either substrate-binding site. Moreover, to allow for the increase in acidity of each set of protonated groups, on returning from the ternary to the binary complexes or to the free enzyme (i.e., at 25 °C, p$K_\kappa \rightarrow$ pK_{H_2} of 5.7 to 5.2; or p$K_\kappa' \rightarrow$ pK_H' of 9.1 to 8.2; see Table II), one possibility would be to permit hydrogen bonding of the thiol group to either an adjacent imidazole or to an ϵ-NH_2 group. From Table IV, the energetics of the system in the "transition" state will provide a maximum potential-energy barrier of either 5 or 17 kcal/mol in either the acidic neutral range or in the basic pH range, respectively. A summary of the mechanistic *ideas* postulated for the mode of action of the D-glucose-6-phosphate dehydrogenase is presented in Figure 5. For simplicity, the mechanism is restricted to the acidic and neutral range, but replacement of the imidazole group with an adjacent ϵ-amino group would permit a similar mechanism to take place in the basic region. Thus, it is presumed that, after the formation of an $HE{\cdot}A^-{\cdot}B^-$ ternary complex (with one proton dissociated in the active center, i.e., from H_2E to yield HE), an hydrogen-bonded thiol–imidazole (unprotonated) grouping acts as the hydrogen atom(s) acceptor system and transferring agent from carbon 1 (and its anomeric hydroxyl group) of β-D-glucose 6-phosphate^{2-} to NADP^{3-}, to eventually result in the "transition state" depicted in Figure 5 and with the liberation of an hydrogen ion. The formation of the ternary complex $HE{\cdot}C^-{\cdot}D^-$ proceeds then with the final liberation of the products, NADPH^{4-} and the δ-lactone of 6-phosphogluconate^{2-}.

With the outlines now discernible on the mode of action of glucose-6-phosphate dehydrogenase, the way appears open for further and more detailed mechanistic probing.

Appendix

The following refer to Figure 4.

A. Lowest Set of Plots: log $V_{max,app}$ vs. pH; solid curves drawn according to eq 31 and 32 in logarithmic form. At 25 °C, theoretical curve was computed from logarithmic form of:

$$V_{max,app}(overall) = \frac{420}{1 + \frac{(H^+)}{2.047 \times 10^{-6}} + \frac{8.395 \times 10^{-10}}{(H^+)} + \frac{905}{1 + \frac{(H^+)}{8.395 \times 10^{-10}} + \frac{(H^+)^2}{1.7185 \times 10^{-15}}}}$$

At 32 °C, theoretical curve was computed from logarithmic form of:

$$V_{max,app}(overall) = \frac{525}{1 + \frac{(H^+)}{2.49 \times 10^{-6}} + \frac{1.2735 \times 10^{-9}}{(H^+)} + \frac{1810}{1 + \frac{(H^+)}{1.2735 \times 10^{-9}} + \frac{(H^+)^2}{3.171 \times 10^{-15}}}}$$

B. Middle Set of Plots: $-\log \bar{K}_{B,app}(overall)$ vs. pH and $-\log \bar{K}_{A,app}(overall)$ vs. pH; solid curves drawn according to eq 33-36 in logarithmic form. At 25 °C, theoretical curves were computed from logarithmic forms of:

$$\bar{K}_{B,app}(overall) = (1.153 \times 10^{-5})\left(1 + \frac{(H^+)}{8.138 \times 10^{-7}}\right) \times \left\{\frac{\left(1 + \frac{(H^+)}{5.902 \times 10^{-6}} + \frac{6.401 \times 10^{-9}}{(H^+)}\right)}{\left(1 + \frac{(H^+)}{2.047 \times 10^{-6}} + \frac{8.395 \times 10^{-10}}{(H^+)}\right)} + \frac{\left(1 + \frac{(H^+)}{6.410 \times 10^{-9}} + \frac{(H^+)^2}{3.7779 \times 10^{-14}}\right)}{\left(1 + \frac{(H^+)}{8.395 \times 10^{-10}} + \frac{(H^+)^2}{1.7174 \times 10^{-15}}\right)}\right\}$$

$$\bar{K}_{A,app}(overall) = (3.14 \times 10^{-6})\left(1 + \frac{(H^+)}{7.413 \times 10^{-7}}\right) \times \left\{\frac{\left(1 + \frac{(H^+)}{5.902 \times 10^{-6}} + \frac{6.401 \times 10^{-9}}{(H^+)}\right)}{\left(1 + \frac{(H^+)}{2.047 \times 10^{-6}} + \frac{8.395 \times 10^{-10}}{(H^+)}\right)} + \frac{\left(1 + \frac{(H^+)}{6.401 \times 10^{-9}} + \frac{(H^+)^2}{3.779 \times 10^{-14}}\right)}{\left(1 + \frac{(H^+)}{8.395 \times 10^{-10}} + \frac{(H^+)^2}{1.7174 \times 10^{-15}}\right)}\right\}$$

At 32 °C, theoretical curves were computed from logarithmic form of:

$$\bar{K}_{B,app}(overall) = (1.473 \times 10^{-5})\left(1 + \frac{(H^+)}{8.318 \times 10^{-7}}\right) \times \left\{\frac{\left(1 + \frac{(H^+)}{3.733 \times 10^{-6}} + \frac{7.460 \times 10^{-9}}{(H^+)}\right)}{\left(1 + \frac{(H^+)}{2.49 \times 10^{-10}} + \frac{1.2735 \times 10^{-9}}{(H^+)}\right)} + \frac{\left(1 + \frac{(H^+)}{7.46 \times 10^{-9}} + \frac{(H^+)^2}{2.7848 \times 10^{-14}}\right)}{\left(1 + \frac{(H^+)}{1.2735 \times 10^{-9}} + \frac{(H^+)^2}{3.171 \times 10^{-15}}\right)}\right\}$$

$$\bar{K}_{A,app}(overall) = (5.682 \times 10^{-6})\left(1 + \frac{(H^+)}{7.413 \times 10^{-7}}\right) \times \left\{\frac{\left(1 + \frac{(H^+)}{3.733 \times 10^{-6}} + \frac{7.460 \times 10^{-9}}{(H^+)}\right)}{\left(1 + \frac{(H^+)}{2.49 \times 10^{-6}} + \frac{1.2735 \times 10^{-9}}{(H^+)}\right)} + \frac{\left(1 + \frac{(H^+)}{7.46 \times 10^{-9}} + \frac{(H^+)^2}{2.7848 \times 10^{-14}}\right)}{\left(1 + \frac{(H^+)}{1.2735 \times 10^{-9}} + \frac{(H^+)^2}{3.171 \times 10^{-15}}\right)}\right\}$$

C. Upper Set of Plots: $-\text{Log}\ (\bar{K}_{B,app}(overall)/V_{max,app}(overall))$ vs. pH and $-\log\ (\bar{K}_{A,app}(overall)/V_{max,app}(overall))$ vs. pH; solid lines drawn from expressions derived from eq 31-36 and from the above numerical forms of $(\bar{K}_{B,app}(overall)/V_{max,app}(overall))$ and $(\bar{K}_{A,app}(overall)/V_{max,app}(overall))$.

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Identity and Properties of the Chloride Effector Binding Site in Hog Pancreatic α-Amylase†

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ABSTRACT: The Cl^- activated α-amylase from mammalian sources has been shown previously to possess one Cl^- binding site per molecule (Levitzki, A., and Steer, M. L. (1974), *Eur. J. Biochem. 41,* 171). Upon binding of the Cl^- effector the k_{cat} of the amylolytic reaction is increased 30-fold whereas the affinity toward the substrate remains unchanged. In the study presented here we have identified the Cl^- binding site as a single ε-amino group of lysine. The pK of the unique amino group was found to be 9.1, significantly lower than the pH of a free ε-amino group of lysine. This ε-NH_2 group can be blocked by a 2,4-dinitrophenyl group upon treating the enzyme with 2,4-dinitrofluorobenzene at pH 7.9. The dinitrophenylamylase is devoid of Cl^- binding capacity but retains its substrate binding capacity. The dinitrophenylamylase also possesses the basal amylolytic activity characteristic of the unmodified Cl^- free enzyme, indicating that the catalytic machinery of the enzyme is not affected by dinitrophenylation. α-Limit dextrins and maltose which bind to the active site protect the enzyme against dinitrophenylation at least as effectively as the Cl^- effector. These observations indicate that the Cl^- binding lysyl residue is close to the active site and, upon binding, the Cl^- effector induces an enhancement in the catalytic efficiency.

Mammalian α-amylase is known to be activated 30-fold by Cl^- anions (Fischer and Stein, 1960, and references therein). It has been shown recently that Cl^- is an exclusive k_{cat} effector activating the enzyme 30-fold toward starch or the low molecular weight substrate *p*-nitrophenyl maltoside (Levitzki and Steer, 1974). The enzyme was shown to possess one chloride binding site per molecule of enzyme with dissociation constants of 3×10^{-4} M at 25 °C and 1.0×10^{-4} M at 4 °C. Chloride binding induces a subtle conformational change in the enzyme as reflected by the suppression of the exchange of 26 protons and a 240-fold increase in the amylase-Ca^{2+} binding constant, from 8.3×10^8 to 2×10^{11} M^{-1}. The conformational change is minor since it it not detected by spectroscopic measurements on the protein such as circular dichroism and fluorescence of tryptophan moieties or of specific probes attached to the enzyme. It is clear, therefore, that the large rate enhancement induced by the Cl^- effector is due to subtle conformational changes, probably confined to the region of the catalytic center.

In this study we have tried to probe the Cl^- binding site by physicochemical measurements and specific chemical modifications. Furthermore, attempts were made to gain more information concerning the interaction between the Cl^- binding site and the substrate binding site.

Materials and Methods

Undiluted acetone powder of hog pancreas was obtained from Marshall Division Laboratories, Miles. Maltose, sucrose, and lactose, all analytical grade, and shellfish glycogen were obtained from Merck. Soluble starch was obtained from British Drug House and diisopropyl fluorophosphate (iPr_2FP)[1] was from Sigma. $Na^{36}Cl$ and [^{14}C]N_2phF[1] were obtained from Radiochemical Centre. All other purchased chemicals used were of the highest analytical grade available. All solutions were prepared in Corning double distilled water. N^{ϵ}-Dinitrophenyllysine and $N^{\epsilon},N^{\epsilon'}$-dinitrophenylbislysine were prepared by Mr. Israel Jacobson from L-lysine·HCl (Ajimoto, Japan) by reaction with N_2pH-F in the presence of excess $CuCO_3$ according to published procedures (Sanger, 1946; Porter and Sanger, 1948; Greenstein and Winitz, 1961). *o*-Dinitrophenyltyrosine and imidazoledinitrophenylhistidine were kindly donated by Dr. Mati Fridlein from the Department of Organic Chemistry. *S*-Dinitrophenylcysteine was obtained from Mann.

α-Amylase. The enzyme was purified according to the method devised by Loyter and Schramm (1962) with slight modifications. The glycogen of the enzyme–glycogen complex was incubated for 2 h at 30 °C in 0.02 M Pipes[1] containing 3×10^{-3} M NaCl and 5×10^{-4} M $CaCl_2$, pH 6.9, to digest the glycogen. The α-limit dextrins were removed by treatment with Norit A charcoal at pH 8.0–8.5. The Norit A was previously treated with 1.0 N HCl, then with 0.01 M EDTA (pH 7.0) and then thoroughly washed with double distilled water. Enzyme concentration was determined spectrophotometrically using the value $E_{1\%}^{280} = 24.1$ (Hsiu et al., 1964) and the enzyme activity was measured according to Bernfeld (1955) but at 30 °C (Loyter

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[1] Abbreviations used are: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; N_2ph-F_2, 1,5-difluoro-2,4-dinitrobenzene; N_2ph, dinitrophenyl; iPr_2-FP, diisopropyl fluorophosphate; pipes, piperazine-N,N′-bis(2-ethanesulfonic acid).