Allosteric Interactions of Yeast Pyruvate Kinase as a Function of pH*

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ABSTRACT: The pH dependencies of the kinetic parameters characterizing the interaction of yeast pyruvate kinase and the substrate phosphoenolpyruvate were investigated in the presence and absence of the allosteric activator fructose 1,6-diphosphate (FDP). From the results series of acid dissociation constants, presented as pK_a values were calculated by a simulation procedure described in detail. While in the presence of fructose 1,6-diphosphate the "interaction coefficient" ($n_{H(FDP)}$) is pH independent, the interaction coefficient in the absence of the activator (n_H) increases as a function of pH to a limiting maximal value $n_H = 2.95$; at pH ≥ 7.0, n_H becomes pH independent. The strong influence of H^+ ions on the allosteric properties of the enzyme is also demonstrated by the pH dependence of the ratio of the half-saturating substrate concentration in the absence of fructose 1,6-diphosphate to that in the presence of the activator, which gives a curve analogous to that of the interaction coefficient (n_H). It is concluded that the equilibrium between a state of higher affinity and a state of lower affinity of the enzyme toward phosphoenolpyruvate is shifted to the state of higher affinity by protonization of a group with a $pK_a = 5.35$. The divergent course of the pH dependence of the half-saturating substrate concentration in the presence and absence of fructose 1,6-diphosphate at pH <7.0 can be directly related to these effects and is not due to changes in the binding properties of the enzyme for substrates, caused by fructose 1,6-diphosphate. From the pH dependence of the half-saturating concentration in the presence of fructose 1,6-diphosphate it follows that two groups with $pK_{a1} = 5.45$ and $pK_{a2} = 9.0$ are involved in the binding process; the latter is also found for the enzyme not activated by fructose 1,6-diphosphate. It is excluded that these pK_a values are due to ionizing groups of the substrates.

The maximum velocity gives a bell-shaped pH dependence with a maximum at pH 6.3, which is not significantly altered by the activator fructose 1,6-diphosphate.

In earlier communications (Haeckel and Hess, 1966; Haeckel *et al.*, 1968; Wieker *et al.*, 1969) we described the allosteric properties of yeast pyruvate kinase.[1] In these studies an influence of H^+ ions upon the cooperativity of the enzyme was indicated by the fact that the maximum in the pH profile of initial velocities is shifted to higher pH values with increasing concentration of the substrate PEP.[1] Also there were significant differences between the profiles in the absence and presence of the activator FDP, as well as a strong pH dependence of the activation of the enzyme by FDP (Haeckel and Hess, 1966; Haeckel *et al.*, 1968). It has also been reported by Rozengurt *et al.* (1969) that H^+ ions affect the allosteric properties of liver pyruvate kinase (type L).

In this paper, following a preliminary communication (Wieker *et al.*, 1970a), we present a more detailed investigation of the pH dependence of the kinetic parameters. It is demonstrated that the H^+ ion not only influences the catalytic and binding properties of yeast pyruvate kinase, but also has to be regarded as an allosteric modifier which shifts the equilibrium from a state of lower affinity (E_A) to a state of higher affinity (E_B) of the enzyme.[2]

Materials and Methods

All chemicals used were p.a. grade and purchased from E. Merck AG, Darmstadt, if not stated otherwise. Lactate dehydrogenase (EC 1.1.1.28), Na_3ADP, tricyclohexylammonium phosphoenolpyruvate, Na_3FDP, and Na_2NADH were bought from C. F. Boehringer & Soehne GmbH, Mannheim-Waldhof, β,β-dimethylglutaric acid from Fluka AG, Buchs SG, Lab-Trol from Dade Reagents Inc., Miami.

Pyruvate kinase of brewer's yeast (*Saccharomyces carlsbergensis*, Hochhefe, Kronenbrauerei Dortmund) was isolated as described earlier with a specific activity of about 200 μmoles min^{-1} (mg of protein)$^{-1}$ under standard assay conditions (Bischofberger *et al.*, 1970). Protein determination was performed according to (Beisenherz *et al.*, 1953), using Lab-Trol as a standard.

pH measurements were carried out with a pH meter 26, Radiometer, Copenhagen, equipped with a glass electrode G 2222C and a calomel electrode K 4112.

Kinetic measurements were performed in a lactate dehydrogenase coupled test at 25°, using an Eppendorf photometer with a filter selecting the 366-nm band as measuring wavelength, in the following medium: 0.1 M KCl, 50 mM $MgSO_4$, 10 mM ADP, 0.25 mM NADH, and 0.1 mg/ml of lactate dehydrogenase (dialyzed against 0.1 M KCl). The concentration of PEP was varied from 5×10^{-5} to 3×10^{-2} M. For activation of the enzyme, 5 mM FDP was added. All substances were dissolved in the following buffers. A β,β-dimethylglutaric acid (0.05 M)–Tris (0.1 M) buffer was used from pH 5.0 to 9.0 and a glycine (0.1 M) buffer was used at pH 9.0 and 9.5. For control, measurements at pH 9.0 were performed with both buffers. The final volume of the assay mixture was 2.0 ml. The measurements were started by addi-

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; pyruvate kinase, ATP:pyruvate phosphotransferase (EC 2.7.1.40).

[2] Symbols not defined in the text: (E_A) = state of lower affinity; (E_B) = state of higher affinity; v = initial velocity/enzyme concentration; V_{max} = maximal velocity/enzyme concentration; $K_{0.5}$ = substrate concentration giving $v = V_{max}/2$; (S) = substrate concentration; n_H = interaction coefficient; K_{a1}, K_{a2} = acid dissociation constants. Parameters obtained in the presence of FDP are indicated by (FDP) *e.g.*, $K_{0.5(FDP)}$.

TABLE I: Kinetic Parameters of Yeast Pyruvate Kinase of the Substrate PEP, in the Absence and Presence of FDP (5 mM), at Different pH.

pH	n_H	$n_{H(FDP)}$	$K_{0.5}$ ($\times 10^3$ M^{-1})	$K_{0.5(FDP)}$ ($\times 10^4$ M^{-1})	$K_{0.5}/K_{0.5(FDP)}$	V_{max}[a]	$V_{max(FDP)}$[a]
5.0	1.68	1.05	1.17	5.34	2.2	59	75
5.5	2.14	1.19	1.23	2.40	5.1	127	145
6.0	2.57	1.09	2.11	2.41	8.8	190	229
6.5	2.70	1.02	2.91	2.10	13.9	165	220
7.0	2.95	1.13	3.48	1.75	19.9	141	200
7.5	2.97	1.10	3.47	1.59	21.8	104	143
8.0	2.93	0.93	3.00	1.66	18.1	90	111
8.5	2.88	0.90	2.35	0.94	25.0	59	54
9.0	3.00	0.95	2.06	0.60	34.3	38	31
9.5	2.99	1.12	1.35	0.57	23.6	14	13

[a] μmoles min^{-1} (mg of protein)$^{-1}$.

tion of 10 μl of diluted pyruvate kinase solution. In separate experiments it was shown that the indicating NADH system does not influence the reaction of pyruvate kinase under these conditions and that pyruvate kinase was stable over the whole pH range investigated.

The concentrations of ADP and $MgSO_4$ were chosen to ensure saturation of the enzyme with this ligand system under all experimental conditions. From the dissociation constants of ADP and the ADP–Mg complex (Sillen and Martell, 1964) it was calculated that the concentration of the dominant species, *i.e.*, the ADP–Mg complex is 8.8 mM at pH 5.0 and 9.9 mM at pH 9.5. Thus, the pH-dependent alterations in the concentration of the complex are only $\pm 6\%$ over the whole pH range investigated (see also Discussion).

All calculations were performed on computer IBM 360-44 level H of 32 K bytes of system 360 memory. For the determination of kinetic parameters, a special-fitting program was used (Wieker *et al.*, 1970b).

Experimental results

At different pH values, the reaction velocities were measured as a function of PEP concentration at constant levels of ADP and magnesium ions (see Methods), both in the absenc and presence of the allosteric activator FDP. Thus, a serie of 20 saturation curves (v *vs.* (S)),[2] each consisting of 25 v values, were obtained. These were sigmoidal in the absence of FDP, and the degree of sigmoidicity was pH dependent. In the presence of FDP the curves were hyperbolic over the whole pH range.

From the saturation curves the kinetic parameters n_H, $K_{0.5}$, and V_{max} were obtained by use of the fitting procedure according to

$$v = \frac{V_{max}}{1 + \left(\frac{K_{0.5}}{(S)}\right)^{n_H}} \quad (1)$$

With the definitions given (for further details, see Appendix A), this equation describes such v *vs.* (S) curves without any preinterpretation by models for allosteric enzymes (Wieker *et al.*, 1970b). The various parameters characterizing the interaction of pyruvate kinase and PEP were computed with a

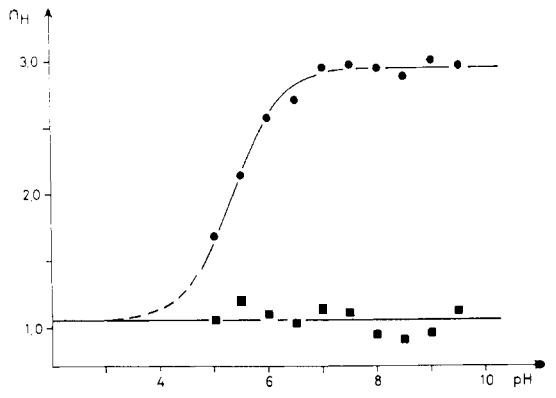

FIGURE 1: pH dependence of n_H. (●) No FDP, (■) 5 mM FDP. The curve of n_H is computed on the basis of eq 4 by the method described in the text; the straight line of $n_{H(FDP)}$ represents the mean value of all $n_{H(FDP)}$. The inflection point of n_H represents the pK_a value (Table II). For dotted line, see text.

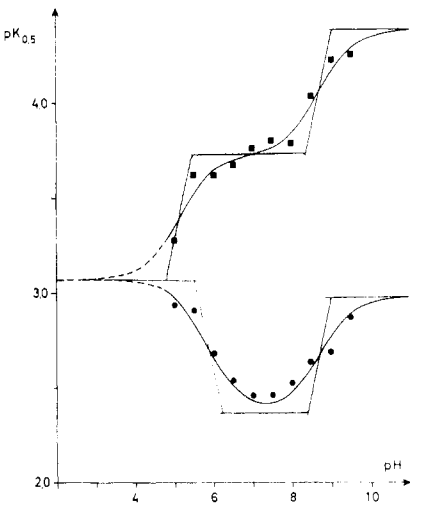

FIGURE 2: pH dependence of p$K_{0.5}$. (●) No FDP, (■) 5 mM FDP. The curves are computed on the basis of eq 10. For straight and dotted lines, see text.

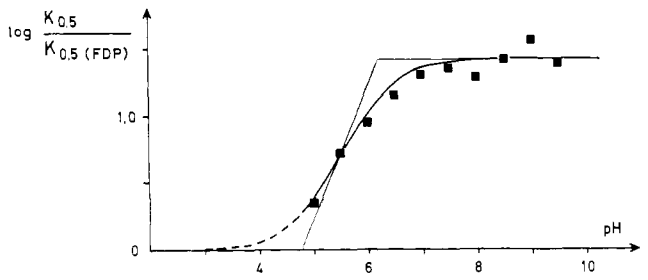

FIGURE 3: pH dependence of log ($K_{0.5}/K_{0.5(FDP)}$). The curve is computed according to eq 10. For straight and dotted lines, see text.

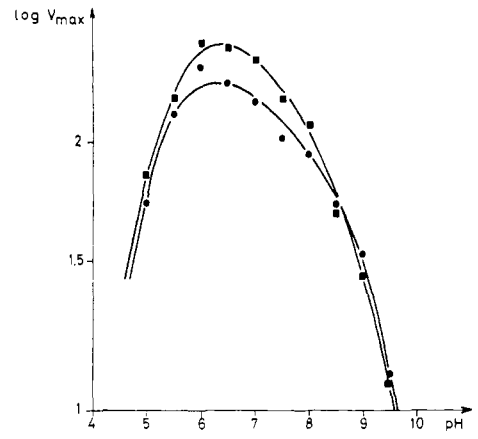

FIGURE 4: pH dependence of log V_{max}. (●) No FDP, (■) 5 mM FDP. The curves are arbitrarily drawn (see text).

mean standard deviation of less than ±5%. They are summarized in Table I and plotted as n_H (Figure 1), p$K_{0.5}$ (Figure 2), log $K_{0.5}/K_{0.5(FDP)}$ (Figure 3), and log V_{max} (Figure 4) as a function of pH, according to Dixon (1953) and Dixon and Webb (1966). The experimental data in the figures are marked by circles and squares, respectively; the drawn curves are obtained by the computation methods given below. The figures clearly demonstrate that the various parameters of the enzyme are a function of pH over a large range.

Because of instability of the enzyme, no parameters could be determined with sufficient accuracy at pH <5.0. However, as reported earlier (Wieker *et al.*, 1969), the influence of the allosteric activator FDP on the cooperativity of yeast pyruvate kinase toward PEP is characterized by a shift from $n_H > 1$ to $n_{H(FDP)} = 1$ and of $K_{0.5}$ to the lower value of $K_{0.5(FDP)}$. As shown in Figures 1–3, analogous shifts are exerted by H^+ ions, and it can be concluded that H^+ ions diminish the cooperativity of the enzyme analogous to other allosteric effectors, *e.g.*, FDP. Together with the pH independency of $n_{H(FDP)}$ these facts are strong arguments for the following assumptions. (i) At low pH, n_H should asymptotically reach the limiting value of $n_{H(FDP)}$ (Figure 1.) (ii) At low pH, as also indicated by the data, $K_{0.5}$ should reach the same value as $K_{0.5}$ (Figure 2), *i.e.*, $K_{0.5}/K_{0.5(FDP)} = 1$ (Figure 3). These assumptions lead to the extrapolations shown by dotted lines in Figures 1–3. Thus, the principal curvatures of the pH dependencies can now be visualized and treated by the following procedure.

Mathematical Treatment and Analysis of the Results

In principle we apply the system of Dixon *et al.* (1966), derived for the interpretation of pH dependencies of enzymes with Michaelis–Menten characteristics. However, the extension of these methods to allosteric enzymes needs some modifications which are given below. The graphical method proposed by Dixon *et al.* (1966) leads to erroneous results, if the dissociation constants are not sufficiently separated from each other. Therefore special computer programs were developed to remove these difficulties, and the following procedure was applied satisfactorily.

As demonstrated in Figure 1, a plot of n_H in a nonlogarithmic scale *vs.* pH leads to a curve resembling the titration curve of a monobasic acid (HA), which can be described by

$$HA \overset{K_{a1}}{\rightleftharpoons} A^- + H^+ \tag{2}$$

$$(A^-) = \frac{(A_t)}{f_a} \tag{3}$$

$$f_a = 1 + \frac{(H)}{K_{a1}} \tag{4}$$

From eq 2 the fraction of the total acid (A_t), which is in the anionic form (A^-), can be calculated by eq 3 and eq 4, where K_{a1} is the dissociation constant and (H) stands for the concentration of H^+ ions. According to equation 3, a plot of $1/f_a$ *vs.* pH represents the fraction of (A^-) at each pH between the asymptotes $1/f_a = 0$ at low pH and $1/f_a = 1$ at high pH. In order to fit the data of n_H (Figure 1), the $1/f_a$ scale was adjusted so that $1/f_a$ reaches asymptotically the limiting values of $n_H = n_{H(FDP)} = 1.05$ and $n_H = 2.95$, respectively, as offered by the curve. By inserting different values of K_{a1}, series of $1/f_a$ were computed as a function of pH, until a best fit with pK_{a1} = 5.35 (Table II) was found. This calculated curve is drawn into Figure 1.

Since the data of $n_{H(FDP)}$ are pH independent over the whole pH range tested, the experimental points were fitted by a straight line representing the mean value of all $n_{H(FDP)}$.

A more complex function is necessary to fit the pH dependencies of $K_{0.5}$, $K_{0.5(FDP)}$, and $K_{0.5}/K_{0.5(FDP)}$ (Figures 2 and 3). Assuming that the enzyme exists in two ionizable states, X and Y,[3] and that there is an equilibrium between HX and HY, the following scheme[4] (eq 5) can be produced, where the

$$\begin{array}{ccccc} H_2X^+ & \overset{K_{a1}}{\rightleftharpoons} & HX & \overset{K_{a2}}{\rightleftharpoons} & X^- \\ & & \uparrow\downarrow \tilde{K}_{xy} & & \\ H_2Y^+ & \underset{K_{a3}}{\rightleftharpoons} & HY & \underset{K_{a4}}{\rightleftharpoons} & Y^- \end{array} \tag{5}$$

K_a's are dissociation constants of the protonated species, and $\tilde{K}_{xy} = (HX)/(HY)$ is a pH-independent equilibrium constant.

Analogous to eq 3 and 4 the fractions of HX from total X (X_t) and HY from total Y (Y_t) can be calculated with (X_t) = f_x (HX) and (Y_t) = f_y (HY), where f_x and f_y are given by eq 6 and 7, respectively. The pH-dependent equilibrium con-

[3] In the original Dixon scheme (Dixon and Webb, 1966) X represents the free enzyme, Y the enzyme–substrate complex, and K_{xy} its dissociation constant. In that case, the f_x represents the product of the ionization functions of free enzyme and free substrate.

[4] For greater clarity, free H^+ ions are not written in this scheme.

TABLE II: Acid Dissociation Constants Estimated from Figures 1 to 3 by the Method Described in the Text.

	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
n_H	5.35			
$pK_{0.5}$	5.50	9.00	6.20	8.40
$pK_{0.5(FDP)}$	5.45	9.00	4.80	8.35
Log $(K_{0.5}/K_{0.5(FDP)})$	6.20		4.80	

$$f_x = 1 + \frac{(H)}{K_{a1}} + \frac{K_{a2}}{(H)} \quad (6)$$

$$f_y = 1 + \frac{(H)}{K_{a3}} + \frac{K_{a4}}{(H)} \quad (7)$$

stant K_{xy}[3] is given by eq 8 or in form of the negative logarithm ($pK_{xy} = -\log K_{xy}$) by eq 9. The pH-independent constants,

$$K_{xy} = \frac{(X_t)}{(Y_t)} = \tilde{K}_{xy}\frac{f_x}{f_y} \quad (8)$$

$$pK_{xy} = p\tilde{K}_{xy} + pf_x - pf_y \quad (9)$$

however, cannot be interpreted without further assumptions or enzyme models. In order to apply the most simple interpretation procedure, pH-independent constants were not determined and eq 10 was used for the computer simulation.

$$\phi = pf_x - pf_y \quad (10)$$

Regardless of whether X and Y represent states of different enzyme affinities or enzyme–ligand interactions,[3] eq 10 describes pH dependencies such as shown in Figures 2 and 3. In this case ϕ stands for $pK_{0.5}$, $pK_{0.5(FDP)}$, and log $(K_{0.5}/K_{0.5(FDP)})$, respectively.

By inserting different pK_a values into eq 6 and 7, numerous curves of ϕ were computed as a function of pH, until, by trial and error, the best fits were obtained. The resulting curves are drawn in Figures 2 and 3, and the pK_a values are summarized in Table II. The straight lines with zero- or one-unit slopes in Figures 2 and 3 are produced according to Dixon (1966) with the pK_a values inserted for simulation. The points of intersection represent these pK_a values. The deviation of the minimum of $pK_{0.5}$ from the straight line demonstrates the overlapping of two neighboring pK_a values.

In addition, from eq 11 it follows that the ϕ function representing log $(K_{0.5}/K_{0.5(FDP)})$ is equal to the difference between the ϕ functions representing $pK_{0.5(FDP)}$ and $pK_{0.5}$. In

$$\log \frac{K_{0.5}}{K_{0.5(FDP)}} = pK_{0.5(FDP)} - pK_{0.5} \quad (11)$$

fact, by inserting the eight pK_a values (Table II: $pK_{0.5}$ and $pK_{0.5(FDP)}$) a curve was obtained which proved to be congruent with the curve (Figure 3) obtained by the direct fit according to eq 10 (Table II: log $(K_{0.5}/K_{0.5(FDP)})$). Thus, the validity of the pK_a values presented is further substantiated. Such simulation procedures could not be applied to the pH dependence of V_{max}, because both curves are obviously not symmetrical (Figure 4), indicating that they can be described only by more complex functions. Therefore, the curves in Figure 4 are arbitrarily drawn. However, two pK_a values can roughly be estimated to be in the region of 5.5 and 8.0, respectively.

Discussion

The systematic analysis of the pH dependency of various parameters of yeast pyruvate kinase leads to a number of pK_a values, which must be differentiated with respect to their structural location within the enzyme or the substrate molecules.

Since the asymmetry of the V_{max} *vs.* pH curves (Figure 4) prevents the determination of pK_a values, it does not seem to be reasonable to discuss the pH dependency of V_{max} in more detail. However, it should be pointed out, that the similarity of the pH dependencies of V_{max} and $V_{max(FDP)}$ indicates that the catalytic functions of yeast pyruvate kinase are not influenced by the allosteric activator FDP.

In contrast to the features of V_{max}, significant differences between the FDP-activated and the nonactivated yeast pyruvate kinase are observed in the pH dependence of the parameters n_H and $K_{0.5}$, which reflect the allosteric properties of this enzyme. As was recently demonstrated (Wieker *et al.*, 1969), the allosteric activator shifts the equilibrium between the states of lower (E_A) and higher (E_B) affinity, causing a shift from $n_H > 1$ to $n_{H(FDP)} = 1$ and a ratio of $K_{0.5}/K_{0.5(FDP)} > 1$. Thus, if $n_H = 1$, the total enzyme is in the state of higher affinity (E_B) and, if n_H reaches its maximum value, the total enzyme is in the state of lower affinity (E_A). Therefore, the pH dependence of n_H and $K_{0.5}/K_{0.5(FDP)}$ reflects the influence of H^+ ions on the cooperativity of pyruvate kinase toward PEP and on the equilibrium between the states E_A and E_B. One may argue that these effects are due to alterations in the ADP–Mg system. However, as pointed out above (see Methods) such alterations are largely eliminated by the experimental conditions. In addition, there is no significant cooperativity of yeast pyruvate kinase toward ADP (Haeckel *et al.*, 1968; Wieker *et al.*, 1970a), so that the significant effects shown cannot be caused by this substrate. Therefore, any pH-dependent alteration of the ADP–Mg system must exert the same effects upon both the FDP-activated and the nonactivated enzyme. The data reported here clearly demonstrate that this is not the case. Thus, there can be no doubt that it is the H^+ ion which diminishes the cooperativity and shifts the equilibrium from the E_A to the E_B state.

Because cooperativity and binding are overlapping in the pH dependence of $K_{0.5}/K_{0.5(FDP)}$, the resulting pK_a values cannot be interpreted at present. However, from the pH dependence of n_H it follows that the E_A state is converted into the E_B state by protonation of a group of the enzyme with a $pK_a = 5.35$ in the following way:

$$E_B \rightleftharpoons E_A + H^+$$

Moreover, from the facts that $n_{H(FDP)}$ is pH independent and that $K_{0.5}$ and $K_{0.5(FDP)}$ merge asymptotically at lower pH, it can be concluded that pyruvate kinase is converted by FDP and H^+ ions into states, which are identical with respect to the binding of PEP. However, it does not necessarily follow that both states are also identical with respect to their total conformations. The same conclusions are obtained if the analysis of the pH dependence of n_H is based on the intrinsic parameters determining the interaction coefficient, as derived in Appendix B.

From $n_{H(FDP)} \simeq 1$ it follows that pyruvate kinase is totally converted into the state of higher affinity (E_B) by the allosteric activator FDP. Therefore the pH dependence of $K_{0.5(FDP)}$ should not be influenced by cooperative effects and therefore reflect the binding of PEP to the E_B state of the enzyme. Remembering that in this case a hyperbolic *v vs. s* characteristic is obtained, it seems to be justified to apply the interpretation of Dixon and Webb (1966) for the FDP-activated pyruvate kinase. Thus, it follows from the pH dependence of the $pK_{0.5(FDP)}$ that in the state of higher affinity two ionizing groups with $pK_{a1} = 5.45$ and $pK_{a2} = 9.0$, respectively, are connected with the binding process and become more acidic in the enzyme–substrate complex, namely, $pK_{a3} = 4.80$ and $pK_{a4} = 8.35$.

At pH <7.0 cooperativity and binding of substrate are overlapping in the pH dependence of $K_{0.5}$. Therefore, no conclusions can be drawn about the pK_a values in this region, namely, $pK_{a1} = 5.5$ and $pK_{a3} = 6.2$. In the alkaline region, however, where the cooperativity is pH independent, once more a group with $pK_{a2} = 9.0$ is found and shifted to $pK_{a4} = 8.4$ in the enzyme–substrate complex. These results indicate that the group with $pK_a = 9.0$ is involved in the binding of PEP, which becomes more acidic in the enzyme–substrate complex, regardless whether the pyruvate kinase is activated by FDP or not.

As pointed out by Dixon and Webb (1966), it is impossible to distinguish, whether pK_{a1} and pK_{a2} of eq 5 reflect the ionizations of the free enzyme or the free substrate.[3] However, in contrast to the ionizations of the enzyme, the pK_a values of the free substrate can be determined, uneffected by neighboring groups. The pK_a values of the phosphate group have been found to be in the region of 6.35–6.40 and the carboxyl groups of the substrate in the region of 3.4–3.5 (Wold and Ballou, 1957). These pK_a values obviously do not fit with the $pK_{a1} = 5.35$, $pK_{a1} = 5.45$, and $pK_{a2} = 9.0$ found in our experiments. Although there is no evidence that the less stable PEP–magnesium complex functions as the substrate of yeast pyruvate kinase, it should be noted that the pK_a value of the phosphate group will be smaller in this complex than in the free PEP. However, binding of this complex must either cause a deletion of the corresponding pK_a value or cause a shift to a higher pK_a value in the enzyme–substrate complex. Both considerations contradict to the results. Therefore, it must be concluded that the pK_a values presented in Table II do not reflect ionizations of the substrate but correspond to ionizing groups of the enzyme.

At present it seems to be too speculative to correlate these pK_a values with amino acid residues of the active center. However, the $pK_{a2} = 9.0$ points to a cysteine residue. In fact, we have observed in a study of the reactivity of pyruvate kinase with SH reagents that a cysteine residue plays an important role in pyruvate kinase activity (H. J. Wieker and B. Hess, in preparation).

In the case of liver pyruvate kinase (type L), Rozengurt *et al.* (1969) also found an influence of H^+ ions on the cooperativity of this enzyme toward PEP, causing an increase of n_H and $K_{0.5}$ at pH >7 with somewhat different properties. Since these authors present no details with respect to the properties of $K_{0.5(FDP)}$ and restricted their experiments to a smaller pH range (5.9–8.3), a comparison of their results to our conclusions does not seem to be justified.

We stress the generality of our observations with respect to controlling functions of H^+ ions in the conformational states of proteins. We have pointed out earlier (Haeckel *et al.*, 1968) that the pH dependence of the allosteric properties of isocitrate dehydrogenase observed by Klingenberg *et al.* (1965) might be based on the same properties described here for pyruvate kinase. Furthermore, the well-known pH dependence of the conformational state of hemoglobin must obviously be mentioned (Wyman, 1968).

The physiological significance of the pK_a values of yeast pyruvate kinase may be regarded with respect to possible changes in the intracellular pH of the yeast cell (Kotyk, 1963) which together with the relationship between a varying FDP concentration and the activity of pyruvate kinase during oscillatory (Hess *et al.*, 1969) and steady states (Barwell *et al.*, 1971) of yeast glycolysis could exert a strong control.

Appendix A

For the analysis of sigmoidal *v vs. s* curves, in many cases the Hill equation (eq A1), linearized in eq A2, is used, which is more familiar than eq 1 used for the analysis of the data

$$v = \frac{V_{max}}{1 + \frac{K}{(S)}} \tag{A1}$$

$$\log \frac{v}{V_{max} - v} = n_H \log (S) - \log K \tag{A2}$$

presented above. The constant K is the product of all dissociation (Michaelis) constants for the separate binding steps (Atkinson *et al.*, 1965). The substrate concentration, $(S_{0.5})$, which is required for half-maximal velocity, can be derived from eq A1 and A2 with $v = V_{max}/2$ or $\log (v/(V_{max} - v)) = 0$, respectively:

$$(S_{0.5}) = \sqrt[n_H]{K} \tag{A3}$$

Using the symbol $K_{0.5}$ instead of $(S_{0.5})$ (see definitions[2]), *i.e.*, $K_{0.5} \equiv (S_{0.5})$, and replacing K in eq A1 by eq A3, eq A1 becomes

$$v = \frac{V_{max}}{1 + \left(\frac{K_{0.5}}{(S)}\right)^{n_H}} \tag{A4}$$

which is identical with eq 1 given above. Equation A4 differs from eq A1 only by the definitions of the parameters K and $K_{0.5}$. However, the analysis of sigmoidal *v vs.* (S) curves according to eq A4 (eq 1) has some advantages: (i) $K_{0.5}$ is numerically equal to the substrate concentration which can often be visualized directly from a *v vs.* (S) curve at $v = V_{max}/2$ or from a Hill plot at $\log (v/(V_{max} - v)) = 0$. Although eq A2 is presented as the basic equation, some investigators identified K with $(S_{0.5})$, visualized from a Hill plot. Thus, they changed the definition of K to that of $K_{0.5}$, *i.e.*, they really used eq A4. (ii) The parameters V_{max}, n_H, and $K_{0.5}$, but not K, are strongly correlated to the intrinsic parameters of enzyme models, regardless whether sigmoidicity is caused by allosteric interaction or not (an example is given in Appendix B). Thus the analysis of *v vs.* (S) curves is not model oriented (Wieker *et al.*, 1970b).

Equation A4 as well as eq A1 imply simplifications, especially the assumption that n_H is independent of (S). If n_H is a function of (S), the Hill plot cannot be linear over the total range (Rubin and Changeux, 1966); therefore n_H has to be

determined as the slope of the Hill plot at $(S_{0.5}) \equiv K_{0.5}$. However, Hill plots usually are linear in the optimum experimental range, *i.e.*, 0.1–0.9 × V_{max} (Frieden, 1967). The computer program used (Wieker *et al.*, 1970b) ensures the determination of n_H in the linear range in the region of $K_{0.5}$, providing a further interpretation of V_{max}, $K_{0.5}$, and n_H by enzyme models.

Appendix B

Since n_H is a complex function of the intrinsic dissociation constants of the substrate from the different states of affinity, one may argue that the pH dependence of n_H cannot be interpreted directly (see Discussion) but has to be based on the intrinsic parameters. As previously demonstrated (Wieker *et al.*, 1969, publication in preparation) the cooperative behavior of yeast pyruvate kinase toward phosphoenolpyruvate, FDP, and ATP is consistent with the model of Monod, Wyman, and Changeux (Monod *et al.*, 1965). Therefore, the equation of Rubin and Changeux (1966), which correlates n_H with the intrinsic parameters can be applied. This equation, formulated in the manner of Buc and Buc (1967), expresses n_H as a function of the number of binding sites n, the parameter $K_{0.5}$ and the dissociation constants of the E_A–substrate complex K_A and the E_B–substrate complex K_B:

$$n_H = 1 + (n - 1)\frac{(K_{0.5} - K_B)(K_A - K_{0.5})}{(K_{0.5} + K_B)(K_A + K_{0.5})} \qquad (B1)$$

Equation B1 allows to analyze the changes of the parameters, which have to be caused by H^+ ions so that the experimental pH dependence of n_H would result.

As shown in Figure 1, H^+ ions diminish n_H down to the limiting value of 1. From eq B1 it follows, that $n_H = 1$ will result, if at least one term of the numerator becomes zero; and the following cases result. Case I: The condition $(n - 1) = 0$ is fulfilled, if there is only one binding site per enzyme molecule. Whatever the reason may be, the result of such an effect of H^+ ions will be the existence of only one kinetically relevant state. This is equivalent with either case II or case III. Thus, case I is only of mathematical meaning. Case II: For $(K_A - K_{0.5}) = 0$, $K_{0.5}$ increase to K_A as the limiting value. This can only be possible, if H^+ ions shift the equilibrium to the state of low substrate affinity E_A, *i.e.*, inhibition by H^+ ions. However, this inhibition must diminish the activation by FDP and cause a pH dependence of $n_{H(FDP)}$ opposite to that of n_H. This predictions are obviously in contrast to the results, and case II can be ruled out. Case III: For $(K_{0.5} - K_B) = 0$, $K_{0.5}$ has to decrease to K_B as the limiting value. This will be possible, if H^+ ions shift the equilibrium to the state of high substrate affinity E_B, *i.e.*, activation by H^+ ions. Since the equilibrium is shifted to the state of high affinity by FDP too, $n_{H(FDP)}$ cannot be influenced by H^+ ions. These predictions obviously agree with the results (Figure 1–3), *i.e.*, case III is valid. Thus, the results of the indirect interpretation via the intrinsic parameters are identical with those of the direct interpretation of the pH dependence of n_H, given above: H^+ ions act as allosteric activators of yeast pyruvate kinase by transforming the state of low-affinity E_A into the state of high-affinity E_B, analogously to FDP. However, this interpretation too does not allow a decision, whether the FDP- and H^+-activated states are identical with respect to their total conformations.

Acknowledgments

The technical assistance of Mrs. B. Spiekermann and Mr. K. -H. Wüster is gratefully acknowledged. We also appreciate the generous supply of brewer's yeast by the Kronenbrauerei, Dortmund.

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