Studies on the Interaction of Substrate and Monovalent and Divalent Cations with Pyruvate Kinase*

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ABSTRACT: The dissociation constants for the interaction of monovalent (K^+, Na^+, NH_4^+, Rb^+, Cs^+) and divalent cations (Mg^{2+}, Mn^{2+}) as well as substrates with pyruvate kinase have been measured under various conditions by protein difference spectroscopy. In addition the dissociation constants for the monovalent cations at zero and infinite concentrations of Mn^{2+} were determined from kinetic data. The dissociation constants for monovalent cations determined kinetically at zero concentration of Mn^{2+} agree favorably with those determined by difference spectroscopy in 0.1 M TMA^+ (tetramethylammonium), a nonactivating cation. The binding of cations K^+, Na^+, Mg^{2+}, and Mn^{2+} measured in the presence of TMA^+ appears to occur essentially in pairs. A preferred order of binding is suggested in which binding occurs preferentially at one site followed by a rapid binding at a second site such that the concentration of the singly associated form is very small. The dissociation constants for the monovalent cations decrease as the ionic radii of the cation increases. The binding of the divalent activator appears to increase the affinity of the enzyme–substrate complex for the monovalent activator as determined kinetically.

Measured by protein difference spectroscopy the dissociation constant for Mn^{2+} is only slightly affected by the nature of the monovalent cation present. The data thus indicate that the monovalent or divalent activating cations mutually influence the affinities for each other. The dissociation constant for phosphoenolpyruvic acid in the presence of 0.1 M K^+ and 5×10^{-3} M Mn^{2+} (1×10^{-5} M) is at least an order of magnitude smaller than that observed in the presence of 0.1 M TMA^+ (2.5×10^{-4} M). Pyruvate does not elicit a difference spectrum in pyruvate kinase in the absence of activating cations but produces a typical tyrosyl difference spectrum in their presence, permitting the determination of K_D (enzyme–cations–pyruvate) = 2.5×10^{-4} M. Adenosine 5′-diphosphate and adenosine 5′-triphosphate do not perturb the spectrum of the protein either in the presence or absence of activating cations.

Boyer *et al.* (1942) clearly demonstrated that pyruvate kinase had an absolute requirement for the cofactors K^+ and Mg^{2+}. This was the first demonstration of a cofactor role for K^+. Since that time a wide variety of enzymes, some not involving phosphoryl transfer, have been found to require K^+ or other monovalent cations such as NH_4^+ or Rb^+ for maximal activity (Von Korff, 1953; Parks *et al.*, 1957; Happold and Beechey, 1958; Richards and Rutter, 1961; Ramasastri and Blakley, 1962; Bowen and Kerwin, 1954; Happold and Struyvenberg, 1954; Bonting *et al.*, 1963; Bessman and Van Bibber, 1959).

The basis for the activation by monovalent cations is uncertain. Kachmar and Boyer (1953) have noted the similarity of the crystal radii of these activating ions in contrast to the less activating Na^+ and nonactivating Li^+. They suggested that both the active and inactive monovalent cations combine with the same site or sites on the enzyme and that such combinations could logically involve some displacement of adjoining structures. The catalytic activity, they continued, may be associated with a critical amount of such displacement of adjoining structures. Happold and Beechey (1958) suggested, on the other hand, that because of the high concentration of KCl which is required for optimum catalysis, this is probably a nonspecific interaction affecting the organization of the enzyme molecule.

It has previously been reported (Suelter and Melander, 1963) that the interaction of manganous chloride or magnesium chloride with pyruvate kinase in the presence of 0.1 M KCl resulted in the appearance of a protein difference spectrum as detected by the technique of difference spectrophotometry (Laskowski *et al.*, 1956). The difference spectrum arising from this perturbation was used to determine the dissociation constant for the interaction of divalent cation with pyruvate kinase (Suelter and Melander, 1963). This paper describes additional experiments by ultraviolet difference spec-

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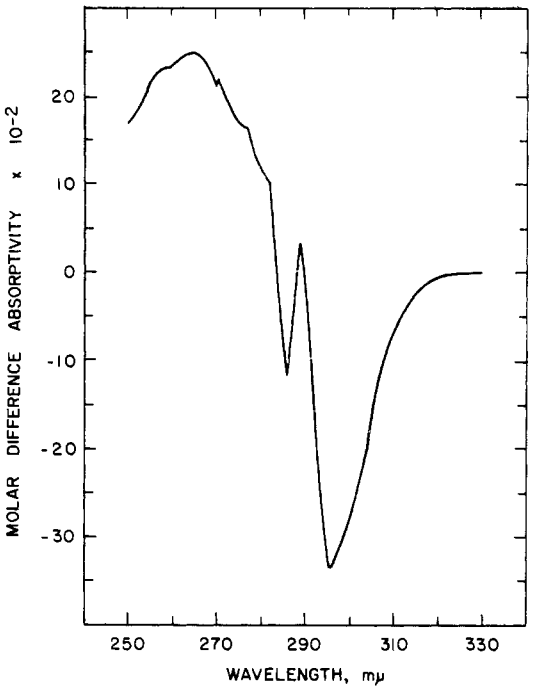

FIGURE 1: Ultraviolet difference spectrum of pyruvate kinase in 0.13 M CsCl and 0.1 M TMACl *vs.* pyruvate kinase in 0.23 M TMACl. Into each of two 3-ml fused-silica cuvets was placed 2 ml of an enzyme solution containing per milliliter: Tris–HCl, pH 7.8, 50 μmoles; TMACl, 100 μmoles; and pyruvate kinase, 2.8 mg. The spectrum was recorded on a Cary Model 15 spectrophotometer after addition of CsCl to the sample cuvets and TMACl to the reference cuvet. The difference spectrum represents that calculated for saturation with CsCl.

troscopy and kinetics to show the interaction of substrates and monovalent and divalent cations with this enzyme.

Materials and Methods

Pyruvate kinase (EC 2.7.1.40) was prepared from fresh frozen rabbit muscle obtained from Pel Freez Biologicals, Inc., Rogers, Ark., by a method previously described (Tietz and Ochoa, 1958), with the exception that recrystallized or Mann Special Enzyme Grade ammonium sulfate was used. All dialysis tubing was boiled in two changes of 10^{-3} M disodium ethylenediaminetetraacetate (EDTA) for 15 min before use. The enzyme was stored in aqueous solution at 50–60 mg/ml rather than as an ammonium sulfate suspension.

The specific activity of each preparation was determined as previously described (Suelter and Melander, 1963) or by the use of a pH-Stat (Radiometer TTT-1/-SBR2/SBU1/TTA31) (Kayne and Suelter, 1965). Since 1 mole of H^+ is taken up for every mole of pyruvic acid liberated, the rate of formation of pyruvic acid can be continuously followed. These activities were variable, ranging from 130 to 250 μmoles/min/mg of enzyme at 25°. When the enzyme is stored in solution at 50–60 mg/ml there is a slow aggregation of inactive protein with a concomitant increase in specific activity. This accounts for some of the variability. Protein concentrations were determined by measuring the absorbance in 0.1 M KCl at 280 mμ using a molar extinction coefficient of 1.28×10^{-5} based on the data of Bücher and Pfleiderer (1955) and on the molecular weight of 237,000 (Warner, 1958).

All cations except the buffer cation were removed from the enzyme by the use of Sephadex G-25 (medium grade). Pyruvate kinase solution (2 ml, 50–60 mg/ml) was placed on a Sephadex column (1.7 × 18 cm) previously equilibrated with 5×10^{-3} M Tris–HCl, pH 8.6, and eluted with the same buffer.

Solutions of monovalent or divalent cations were prepared as chlorides from the reagent grade salts. Manganous chloride solutions were standardized by titration with standardized EDTA using Eriochrome Black T indicator or by oxidation to permanganate (Sandell, 1959).

The data for each titration with cations were obtained with a Beckman DU spectrophotometer equipped with a Gilford cuvet positioner and multiple-sample absorbance recorder. Although the magnitude of the difference spectrum was a linear function of the protein concentration up to at least 6.4 mg/ml, all measurements were made on solutions containing 2.0–3.7 mg/ml in order to conserve enzyme and yet have a large enough difference to make accurate measurements. Corrections for absorption due to tetramethylammonium chloride (TMACl)[1] were made when this absorption represented an appreciable fraction of the difference.

The titrations were performed by the method of Suelter and Melander (1963). Occasionally precipitation occurred during a titration, which could readily be detected by noting changes in absorption at 330 mμ. This problem could usually be alleviated by allowing the diluted protein solution to remain at 25° for 20–45 min and centrifuging prior to a titration.

For the titrations of the enzyme with substrates, the tandem-cell method of Herskovits and Laskowski (1962) was used as described previously (Kayne and Suelter, 1965). A Cary Model 15 spectrophotometer was used to record the substrate-produced difference spectra. Phosphoenolpyruvic acid (PEP), tricyclohexylammonium salt, and adenosine 5′-triphosphate (ATP) were obtained from the Sigma Chemical Co., St. Louis, Mo., and adenosine 5′-diphosphate (ADP) from the Pabst Laboratories, Milwaukee, Wis. Pyruvic acid was obtained from Matheson Coleman and Bell, Norwood, Ohio, and redistilled before use. All solutions were neutralized with tetramethylammonium hydroxide to the required pH when necessary.

[1] Abbreviations used in this work: TMACl, tetramethylammonium chloride; TMA^+, tetramethylammonium cation; PEP, phosphoenolpyruvic acid; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate.

The kinetic experiments shown in Figure 3 were carried out by following the rate of appearance of pyruvate by coupling the reaction catalyzed by pyruvate kinase to the lactic dehydrogenase catalyzed reaction (Bücher and Pfleiderer, 1955) as previously described (Mildvan and Cohn, 1965). Prior to use, both pyruvate kinase and lactic dehydrogenase were separated from ammonium sulfate using Sephadex G-25 as previously described (Mildvan and Cohn, 1965). The tetramethylammonium cation (TMA^+) salt of ADP was prepared by ion-exchange chromatography on Dowex 50-TMA^+.

Results

When the divalent cations Mg^{2+} or Mn^{2+} interact with pyruvate kinase in the presence of 0.1 M K^+, a protein difference spectrum is observed (Suelter and Melander, 1963). This difference spectrum was shown to be due to a change in the environment of a tryptophyl residue(s) brought about by a change in the conformation of the protein molecules (Kayne and Suelter, 1965). When divalent or monovalent cations interact with pyruvate kinase in the presence of 0.1 M TMA^+, a nonactivating cation, a protein difference spectrum is again obtained as shown in Figure 1 for CsCl. This difference spectrum is qualitatively identical with that obtained in the presence of $MnCl_2$ and KCl (Kayne and Suelter, 1965; Figure 3).

When the change in absorbancy at the maximum, 295 mμ, was plotted *vs.* the negative log of the monovalent or divalent cation concentration, a sigmoid curve was obtained as shown for Mg^{2+} in Figure 2A and for K^+ in Figure 2B. The line connecting the experimental data was obtained from eq 1 with $n = 2$. The derivation of this equation is described in the Discussion.

$$n(pK_{av} - pM) = \log \frac{\alpha}{1 - \alpha} \qquad (1)$$

TABLE I: Dissociation Constants for Monovalent and Divalent Cations Determined by Protein Difference Spectrophotometry.[a]

Cation	Radius[b] (A)	n(eq 1)	Dissociation Constant
Mg^{2+}	0.65–0.78	2	$9.1 \pm 0.2 \times 10^{-4}$
Mn^{2+}	0.80	2	$1.0 \pm 0.2 \times 10^{-4}$
Na^+	0.95	2	$2.1 \pm 0.2 \times 10^{-1}$
K^+	1.33	2	$1.2 \pm 0.2 \times 10^{-1}$
Rb^+	1.49	3	$5.0 \pm 0.5 \times 10^{-2}$
NH_4^+	1.49	4	$4.7 \pm 0.6 \times 10^{-2}$
Cs^+	1.69	3	$4.4 \pm 0.4 \times 10^{-2}$

[a] Each titration was performed under the same conditions as noted in the legend for Figure 2A except the concentration of protein varied from 2.0 to 3.7 mg/ml. [b] Univalent crystal radii (Pauling, 1960).

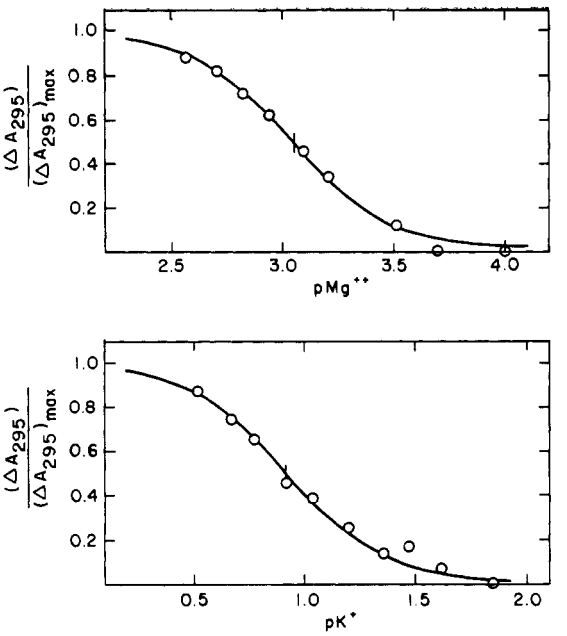

FIGURE 2: pMg^{2+}- (A, upper) and pK^+-dependent (B, lower) titrations of pyruvate kinase. Into each of two 3-ml silica cuvets was placed 2 ml of an enzyme solution containing per milliliter: Tris–HCl, pH 7.8, 50 μmoles; and pyruvate kinase that had previously been passed over Sephadex (G-25) in 5×10^{-3} M Tris–HCl, pH 8.6, 2.9 mg. The titration was performed as previously described (Suelter and Melander, 1963).

Table I lists the dissociation constants for various monovalent and divalent cations determined in the presence of TMACl. It should be noted that data obtained for the interaction of monovalent cations did not always satisfy eq 1 in which $n = 2$. In the case of Rb^+ and Cs^+, $n = 3$, and for NH_4^+, $n = 4$.

To determine whether the binding sites for monovalent cations on pyruvate kinase which are detected by difference spectroscopy are also related to the activity of the enzyme, we have compared the results of binding studies with those of kinetic studies. The kinetic studies exemplified in Figure 3 were analyzed from the following considerations.

If pyruvate kinase is saturated with substrates ADP and PEP, we represent the ternary complex of enzyme–PEP–ADP as ES_1S_2. In the presence of limiting concentrations of monovalent $[M_1]$ and divalent $[M_2]$ activators, the following equilibria exist, assuming Michaelis–Menten kinetics with respect to the metal ions (Mildvan and Cohn 1964, 1965).

$$ES_1S_2 + M_1 \rightleftarrows ES_1S_2M_1; \quad K_1 = \frac{[ES_1S_2][M_1]}{[ES_1S_2M_1]}$$

$$ES_1S_2 + M_2 \rightleftarrows ES_1S_2M_2; \quad K_2 = \frac{[ES_1S_2][M_2]}{[ES_1S_2M_2]}$$

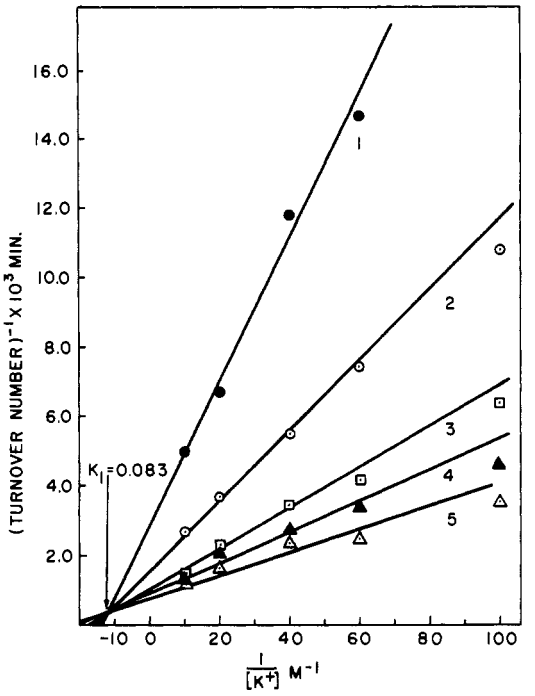

FIGURE 3: Double reciprocal plot of the effect of the monovalent activator (K^+) on the velocity of the pyruvate kinase reaction at various levels of the divalent activator (Mn^{2+}). The reactants were PEP, 6×10^{-4} M; ADP, 1.4×10^{-3} M; $MnCl_2$, curve 1, 1.7×10^{-5} M; curve 2, 3.4×10^{-5} M; curve 3, 6.7×10^{-5} M; curve 4, 8.3×10^{-5} M; curve 5, 1.15×10^{-4} M. In addition each cuvet contained 0.05 M Tris–HCl buffer, pH 7.5; DPNH, 1.5×10^{-4} M; lactate dehydrogenase, 14 μg/ml; and pyruvate kinase, 1.9 μg/ml. The ionic strength was adjusted to 0.14 with TMACl. The reaction was started with $MnCl_2$. The turnover number is defined as the number of moles of pyruvate formed per enzyme site per minute assuming the enzyme to have a molecular weight of 237,000 (Warner, 1958) and two active sites/mole (Mildvan and Cohn, 1965); T = 25°.

$$ES_1S_2M_1 + M_2 \rightleftarrows ES_1S_2M_1M_2;\ K_3 = \frac{[ES_1S_2M_1][M_2]}{[ES_1S_2M_1M_2]}$$

$$ES_1S_2M_2 + M_1 \rightleftarrows ES_1S_2M_1M_2;\ K_4 = \frac{[ES_1S_2M_2][M_1]}{[ES_1S_2M_1M_2]}$$

From the definitions of the equilibrium constants, it is seen that

$$K_1K_3 = K_2K_4 \qquad (2)$$

If the rate is given by the decomposition of the complex $[ES_1S_2M_1M_2]$

$$v = k[ES_1S_2M_1M_2] \qquad (3)$$

and

$$V_{max} = k[ES_1S_2]\left(1 + \frac{[M_1]}{K_1} + \frac{[M_2]}{K_2} + \frac{[M_1][M_2]}{K_2K_4}\right) \qquad (4)$$

Substituting in eq 3 from the definitions of K_1 through K_4 and using eq 2 one obtains the following rate equation.

$$v = \frac{V_{max}}{1 + \frac{K_4}{[M_1]} + \frac{K_3}{[M_2]} + \frac{K_2K_4}{[M_1][M_2]}} \qquad (5)$$

On setting $[M_1] = -K_1$ the velocity becomes independent of $[M_2]$ which holds only at the intersection of lines (Figure 3).

$$v \text{ (at intersection)} = \frac{V_{max}}{1 - \frac{K_4}{K_1}} \qquad (6)$$

K_1 may therefore be found from the intersection of lines (Figure 3). When K_1 is found, K_4 may be determined from eq 6. The K_M of M_1 at a finite concentration of M_2 may be shown from eq 2 and 5 to be

$$K_M(M_1) = \frac{K_1\left(\frac{[M_2]}{K_2} + 1\right)}{\frac{[M_2]}{K_3} + 1} = \frac{K_4\left(1 + \frac{K_2}{[M_2]}\right)}{1 + \frac{K_3}{[M_2]}} \qquad (7)$$

and thus, alternative graphical determinations of K_1 and K_4 may be made by extrapolation of the K_M of M_1 to zero concentration of M_2 and to infinite concentration of M_2, respectively. The determinations of K_2 and K_3 from kinetic data require a knowledge of the concentration of free divalent ion and will not be discussed here.

The results of the kinetic study in which both the K^+ and Mn^{2+} were varied at saturating concentrations of both substrates are shown in Figure 3. The point of convergence of the lines gives the value of K_1, the dissociation constant of K^+ from the enzyme–substrate complex (0.083 M in this experiment). K_4 (0.014 M in this experiment) is then determined by the use of eq 6. The extrapolation methods give very similar values of K_1 and K_4. The K_1 and K_4 values of all of the monovalent activators determined by the same methods using Mn^{2+} as the divalent activator throughout are given in Table II. The K_1 values obtained by kinetics (Table II) are in good agreement with the dissociation constants obtained by difference spectroscopy (Table I).

Table III lists the dissociation constants determined for Mn^{2+} in the presence of various monovalent or divalent cations. The concentration of the monovalent or divalent cation present during a determination was roughly equivalent to the half-maximal saturation indicated by the dissociation constants of the individual cations as listed in Table I. The constants as listed in

TABLE II: Kinetically Determined Dissociation Constants of Monovalent Activators of Pyruvate Kinase.[a]

M^+	Ionic Strength	K_1	K_4	No. of Expts
K^+	0.14	0.10 ± 0.04	0.014 ± 0.002	3
K^+	0.24	0.074 ± 0.020	0.014 ± 0.007	3
Na^+	0.24	0.30 ± 0.03	0.090 ± 0.040	3
NH_4^+	0.24	0.069 ± 0.020	<0.002	3
Cs^+	0.24	0.049 ± 0.013	0.007 ± 0.002	3
Rb^+	0.24	0.050 ± 0.017	0.004 ± 0.002	1

[a] Experimental conditions are as described in the text and in the legend to Figure 3. K_1 and K_4 represent the Michaelis constants of the monovalent activators extrapolated to zero and infinite concentrations, respectively, of the divalent activator.

TABLE III: Dissociation Constants for the Interaction of Mn^{2+} with Pyruvate Kinase in the Presence of Various Cations Determined by Difference Spectrophotometry.[a]

Cation (M)	Radius[b] (A)	Dissociation Constant
Cs^+ (0.05)	1.69	$2.5 \pm 0.9 \times 10^{-5}$
Rb^+ (0.075)	1.49	$3.3 \pm 0.5 \times 10^{-5}$
NH_4^+ (0.04)	1.49	$3.1 \pm 1.0 \times 10^{-5}$
K^+ (0.1)	1.33	$3.7 \pm 0.6 \times 10^{-5}$
Na^+ (0.1)	0.95	$6.2 \pm 0.9 \times 10^{-5}$
Mn^{2+} (5×10^{-5})	0.80	$6.3 \pm 0.4 \times 10^{-5}$
Mg^{2+} (5×10^{-4})	0.65–0.78	$6.6 \pm 0.7 \times 10^{-5}$

[a] Experimental conditions are as described in the legend of Figure 4. [b] Pauling (1960).

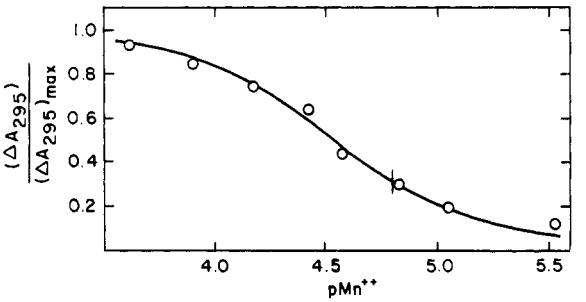

FIGURE 4: pMn^{2+}-dependent titration of pyruvate kinase in the presence of CsCl. The conditions are the same as described for Figure 2A, except that in addition to buffer each enzyme solution contained per milliliter: Sephadexed pyruvate kinase, 3.0 mg; CsCl, 50 μmoles; and TMACl to a final ionic strength of 0.14.

Table III are sufficiently small such that eq 1 with $n = 1$ cannot be used unless suitable corrections for bound Mn^{2+} at every point in a titration are applied. In each case, however, the dissociation constant is described by

$$K_D = \frac{[\text{enzyme}][\text{Mn}]}{[\text{enzyme–Mn}]} \quad (8)$$

where [enzyme] and [enzyme–Mn] represent the concentration of *free* and *complexed* active sites, respectively.

Representative data for the determination of the dissociation constants for Mn^{2+} in the presence of various cations as listed in Table III are shown in Figure 4 for CsCl. The line connecting the points was calculated with eq 8 assuming that one divalent cation binds/active site. This was done by trial and error and was easily accomplished by programming the calculations on a CDC 3600 computor. Since it was not always possible to experimentally obtain the theoretical maximum change in absorption at 295 mμ, several approximate values (5–8) were assumed for computor programming. A K_D was then calculated for each experimental observation in a determination. Finally the computor was programmed to average the dissociation constants calculated with each assumed maximum change in absorption and to use each average K_D to calculate the points for a theoretical curve. The theoretical curve which best fits the data was selected empirically.

The dissociation constants for Mn^{2+} determined in the presence of Cs^+, Rb^+, NH_4^+, or K^+ are the same within experimental error while those determined in the presence of Na^+, Mn^{2+}, and Mg^{2+} are also the same and roughly twice as large as those noted above. One obtains these same groupings if the atomic radii are considered. While the effect is not large, the data do suggest that the larger the size of the cation the smaller the dissociation constant for Mn^{2+}. This effect may be due partly to the effect of the size of the cation and partly due to a competition between the cations.

When the substrates, PEP or pyruvate, interact with the activated or nonactivated forms of the enzyme,

TABLE IV: Dissociation Constants for the Interaction of Substrates with Pyruvate Kinase in the Presence of Various Cations.[a]

Substrate	Cations Present	pK_D[b]
PEP	0.1 M TMA^+	3.6 ± 0.2
PEP	0.1 M K^+	4.1 ± 0.1
PEP	0.2 M K^+	3.9 ± 0.1
PEP	0.1 M K^+ + 0.005 M Mn^{2+}	5.0 ± 0.1, 4.70[c]
PEP	0.1 M TMA^+ + 0.005 M Mn^{2+}	5.0 ± 0.1
Pyruvate	0.1 M TMA^+	...[d]
Pyruvate	0.1 M K^+	...[e]
Pyruvate	0.1 M K^+ + 0.01 M Mn^{2+}	3.6, 3.77[c]
Pyruvate	0.1 M TMA^+ + 0.005 M Mn^{2+}	3.5 ± 0.1
Pyruvate	0.1 M K^+ + 0.01 M Mg^{2+} pH 8.5	3.10–3.62[f]

[a] Pyruvate kinase at concentrations of 2.0–2.5 mg/ml in 0.05 M Tris–HCl, pH 7.8. [b] Negative log of the dissociation constant. [c] Value obtained by Mildvan and Cohn (1963) from proton relaxation rate measurements. [d] No ultraviolet difference spectrum (250–300 mμ) observed. [e] Difference spectrum too small to determine pK_D. [f] Values obtained by Reynard *et al.* (1961) by the ultracentrifuge method in presence of $MgSO_4$ (0.01 M), KCl (0.1 M), and glycylglycine buffer at pH 8.5 and 2°.

difference spectra typical of tryphtophan and/or tyrosine, as shown previously (Kayne and Suelter, 1965), are produced. Table IV lists the dissociation constants obtained for these substrates under a variety of conditions. Those constants determined in the presence of 0.1 M KCl and 0.005 M $MnCl_2$ are in reasonable agreement with those obtained from proton relaxation rate measurements (Mildvan and Cohn, 1963).

No significant protein ultraviolet difference spectrum was detected when the activated or nonactivated enzyme was titrated with ADP or ATP. In these cases, a nucleotide concentration of 6×10^{-4} M [at least six times the reported dissociation constant (Mildvan and Cohn, 1963)] was made possible by the use of 2-mm path length cuvets in tandem. The protein concentration was approximately five times that used when working with the 1-cm cuvets.

Discussion

When the dissociation constant for Mn^{2+} is determined in the presence of 0.05 M CsCl as shown in Figure 4, the data satisfy eq 8 when it is assumed that 1 mole of Mn^{2+} interacts at each catalytic site (Mildvan and Cohn, 1965). The excellent fit of the data shown in Figure 4 with eq 8 indicates that each catalytic site can be considered as a separate entity and, thus, in the presence of 0.05 M CsCl the binding of Mn^{2+} at one site does not effect the affinity or binding process for Mn^{2+} at the other site. Entirely analogous results have been obtained with all monovalent and divalent cations used as activators noted in Table III. This conclusion is compatible with the linear kinetic plots obtained by variation of the concentration of the divalent activators (Mildvan and Cohn, 1965) and substrates (Reynard *et al.*, 1961).

The interaction of cations with pyruvate kinase in the presence of 0.1 M TMA^+, a nonactivating cation, however, is not as easily interpreted. When the interaction of Mg^{2+} or K^+ was studied in the presence of TMACl, the data of Figure 2A and 2B were obtained. The line connecting the points in each case is described by eq 1 with $n = 2$ and represents the theoretical curve expected for a diprotic acid in which the 2 protons dissociate simultaneously with no detectable amount of an intermediate singly dissociated form (Schwarzenbach and Sulzberger, 1943; Maier and Metzler, 1957).

Thus, when activating cations are added to pyruvate kinase in the presence of 0.1 M TMACl rather than 0.1 M KCl, 2 moles of the added cation interact with the enzyme in producing the difference spectrum. One possible explanation for this finding is that for each catalytic site there is a site for monovalent cation and one for divalent cation, but in the absence of one or the other, the sites are not specific and divalent or monovalent cations will bind at both sites. There is no indication of two separate dissociation constants for K^+ or Mg^{2+}, that is, one describing interaction at the divalent site and one describing interaction at the monovalent site. Furthermore, the data do not satisfy the case in which the dissociation constants at both sites are assumed to be identical and that both sites must be occupied before a difference spectrum is observed.

If the binding is represented by the following successive reactions

$$E + M \rightleftharpoons EM_I \qquad K_I = \frac{[E][M]}{[EM_I]}$$

$$EM + M \rightleftharpoons EM_{II} \qquad K_{II} = \frac{[EM][M]}{[EM_{II}]}$$

$$EM_{n-1} + M \rightleftharpoons EM_n \qquad K_n = \frac{[EM_{n-1}][M]}{[EM_n]}$$

and if $K_I > K_{II} > K_n$, then

$$pK_I + pK_{II} \ldots + pK_n - np[M] = \log \frac{\alpha}{1 - \alpha}$$

where $\alpha = \Delta A/\Delta A_{max}$ (Hermans *et al.*, 1960); $\Delta A/\Delta A_{max}$ represents the fraction of enzyme–metal complex. From this eq 1 follows.

Thus one interpretation consistent with the fact that the data in Figures 2A and 2B can be described by eq 1 is one in which the dissociation constant for the second mole of cation (K_{II}) is less than the dissociation constant for the first mole of cation. The measured dissociation constant as shown by eq 1 is equal to the geometric mean of the dissociation constants of the interacting sites.

In addition to the above, the data of Table I suggest that the ionic radii of the monovalent cations affect the dissociation constants for the interaction of monovalent cations in the presence of TMA^+.

Analogous to the above interpretation of the data given in Table I for $n = 2$, the data for Rb^+ and Cs^+, where $n = 3$, and for NH_4^+, $n = 4$, may indicate successive addition of 3 or 4 moles of cation/catalytic site. The measured dissociation constants are, as shown by eq 1, still interpreted as being equal to the geometric mean of the dissociation constants of the interacting sites. However, it should be pointed out that no direct measurements of the moles of monovalent cation bound per mole of protein have been made. The justification for the interpretation of the data for $n = 2$ is based on the direct measurement of 2 moles of Mn^{2+} bound/catalytic site of pyruvate kinase in the presence of TMA^+ (Mildvan and Cohn, 1964).

The data for the kinetically determined dissociation constants of monovalent activating cations from the enzyme–substrate complex at zero and infinite concentration of Mn^{2+} listed in Table II extend the above conclusions. The dissociation constants for monovalent cations at zero concentration of Mn^{2+} (Table II) agree very favorably with the values obtained by difference spectroscopy noted in Table I. Furthermore, the binding of the divalent activator appears to increase the affinity of the enzyme–substrate complex for the monovalent activator. The affinity for the NH_4^+ ion was increased by the largest factor (>35) such that the dissociation constant for the NH_4^+ ion from $ES_1S_2M_2$ was below the level of detection by the kinetic analysis.

The sum total of these data indicates that the monovalent or divalent activating cations mutually influence the affinities for each other, and that in absence of either one, the other cation binds, essentially in pairs, to produce the difference spectrum. This so-called binding in pairs may be a random, preferred, or compulsory process. However, a compulsory order is unlikely since the binding of either activator can be demonstrated in the absence of the other activator by difference spectroscopy or by kinetics. A preferred order is not only suggested by the data of Figures 2A and 2B but also by the fact that the affinity of the enzyme for the divalent activator is rather insensitive to the monovalent activator, while the affinity of the enzyme for the monovalent activator is quite sensitive to the presence of the divalent activator. Thus, the dissociation constant for K^+ in the presence of 0.1 M TMACl as determined by difference spectroscopy is 0.12 M (Table I), and determined kinetically at zero concentrations of Mn^{2+} is 0.10 M (Table II). However, the constant for K^+ obtained kinetically in the presence of Mn^{2+} ranges from 0.016 M (Mildvan and Cohn, 1964) to 0.011 M (Kachmar and Boyer, 1953). The constant obtained for Mn^{2+} in the presence of 0.1 M TMACl is 1×10^{-4} M (Table I) while that obtained in the presence of 0.1 M KCl is 3.7×10^{-5} M (Table III). These values were obtained at pH 7.8 instead of pH 7.5 as previously reported (Suelter and Melander, 1963). Thus the constant for K^+ in the presence of 0.1 M TMACl is 8–11 times larger than that obtained in the presence of $MnCl_2$, while the value for Mn^{2+} determined in the presence of 0.1 M TMACl is 2–3 times larger than that determined in the presence of 0.1 M KCl. Therefore, the constants obtained for Mn^{2+} under both sets of conditions are in better agreement than those obtained for K^+. Hence, if cation is preferably bound at the divalent site followed by interaction at the monovalent site, the value obtained for Mn^{2+} under both conditions should agree while the constants obtained for K^+ would not. That is, the dissociation constant obtained for K^+ in the presence of 0.1 M TMACl would reflect binding at the divalent site and presumably would be somewhat larger, as is observed. However, since the "agreement" of the dissociation constants for Mn^{2+} in TMACl and in KCl may be fortuitous, a detailed kinetic and stoichiometric analysis of the individual steps of metal binding is required to provide a more critical test of the preferred-order hypothesis.

It might also be pointed out that the rate of dissociation and association of cations from the preferred site of a protein conformation resulting from a metal–protein interaction may be much more rapid than the rate at which this protein conformation reverts back to the configuration prevalent in the absence of cation. Thus the protein with monovalent or divalent cation bound at the preferred site may maintain that configuration sufficiently long after dissociation of the cation to bind the correct cations at both sites and become catalytically active. This is in accord with the suggestion of Weber (1965) that cooperative effects can arise as a result of slow equilibria among the protein forms such that equilibrium among them is not maintained in the course of binding. It may be pointed out here that preliminary work has shown that the conformation change observed when Mn^{2+} is bound is a relatively fast process with a half-time considerably less than 1 sec. Such a mechanism may explain the data observed for the interaction of Rb^+, Cs^+, and NH_4^+ as noted in Table I. An apparent stoichiometry of 3 for Rb^+ and Cs^+ and 4 for NH_4^+ might, in fact, represent an additional coopera-

tive effect with paired binding as observed with Na^+, K^+, Mn^{2+}, and Mg^{2+}.

Activating cations also affect the binding of substrate as shown in Table III. The dissociation constant for PEP in the presence of 0.1 M K^+ and 5×10^{-3} M Mn^{2+} or in the presence of 5×10^{-3} M Mn^{2+} is at least one order of magnitude smaller than that observed in the presence of 0.1 M TMA^+, a nonactivating cation. The dissociation constant observed in the presence of 0.1 M or 0.2 M K^+ is somewhat smaller than that observed in 0.1 M TMA^+. Whether the activating cations participate in the binding of PEP *per se* or whether the conformation of the metal–enzyme complex is more suitable for binding PEP cannot be deduced from these data. The observation that the enzyme in the presence of Mn^{2+} has a higher affinity for PEP than the enzyme in the presence of K^+ is compatible with but does not prove a metal bridge structure E–Mn–PEP (Mildvan and Cohn, 1963).

Pyruvate does not elicit a difference spectrum in pyruvate kinase when added to the enzyme in 0.1 M TMACl. It is not known whether pyruvate does not bind under these conditions or whether the binding does not lead to a difference spectrum. A typical tyrosyl difference spectrum is observed, however, when pyruvate is added in the presence of activating cations, and the dissociation constant of pyruvate agrees with K_D obtained by the ultracentrifuge method (Reynard *et al.*, 1961) and the proton relaxation rate method (Mildvan and Cohn, 1963). These data would indicate that activating cations are essential for binding pyruvate or else, as stated above, the binding of pyruvate in the absence of activating cations does not lead to the difference spectra. This conclusion is supported by the results of the detritiation of tritiated pyruvate by pyruvate kinase as reported by Rose (1960). Loss of tritium required the presence of both monovalent and divalent activating cations.

It is interesting that the other substrates, ADP and ATP, do not perturb the spectrum of the protein either in the presence or absence of activating cations. This could be interpreted in a number of ways relative to the previous conclusions but should await further investigation. In this respect, it should also be noted that Ca^{2+} does not elicit a protein difference spectrum. It has been shown that Ca^{2+} does interact with the enzyme since the apparent dissociation constant for Mn^{2+} is increased at increasing levels of Ca^{2+} (Mildvan and Cohn, 1965; Suelter and Melander, unpublished data), and kinetically Ca^{2+} is competitive with Mn^{2+}-activated pyruvate kinase (Mildvan and Cohn, 1965). Whether or not the interactions discussed in this paper have any physiological significance depends on the relative levels of the physiological univalent and divalent cations *in vivo* and, hence, is beyond the scope of this discussion.

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References

Bessman, M. J., and Van Bibber, M. J. (1959), *Biochem. Biophys. Res. Commun. 1.*, 101.

Bonting, S. L., Caravaggio, L. L., and Hawkins, N. M. (1963), *Arch. Biochem. Biophys. 101*, 47.

Bowen, W. J., and Kerwin, T. D. (1954), *J. Biol. Chem. 211*, 237.

Boyer, P. D., Lardy, H. A., and Phillips, P. H. (1942), *J. Biol. Chem. 146*, 673.

Bücher, T., and Pfleiderer, G. (1955), *in* Methods in Enzymology, Vol. I, Colowick, S. P., and Kaplan, N. O., eds., New York, N. Y., Academic, pp. 435, 440.

Happold, F. C., and Beechey, R. B. (1958), *Biochem. Soc. Symp. 15*, 52.

Happold, F. C., and Struyvenberg, A. (1954), *Biochem. J. 58*, 379.

Hermans, J., Jr., Donovan, J. W., and Scheraga, H. A. (1960), *J. Biol. Chem. 235*, 91.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 3418.

Kachmar, J. F., and Boyer, P. D. (1953), *J. Biol. Chem. 200*, 669.

Kayne, F. J., and Suelter, C. H. (1965), *J. Am. Chem. Soc. 87*, 897.

Laskowski, M., Jr., Widom, J. M., McFadden, M. L., and Scheraga, H. A. (1956), *Biochim. Biophys. Acta 19*, 581.

Maier, G. D., and Metzler, D. E. (1957), *J. Am. Chem. Soc. 79*, 4386.

Mildvan, A. S., and Cohn, M. (1963), Abstracts of the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept. 9–13, p. 82C.

Mildvan, A. S., and Cohn, M. (1964), Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July 26–Aug. 1, p. 322.

Mildvan, A. S., and Cohn, M. (1965), *J. Biol. Chem. 240*, 238.

Parks, R. E., Jr., Ben-Gershom, E., and Lardy, H. A. (1957), *J. Biol. Chem. 227*, 231.

Pauling, L. (1960), The Nature of the Chemical Bond, 3rd ed., Ithaca, N. Y., Cornell Univ. Press, p. 514.

Ramasastri, B. V., and Blakley, R. L. (1962), *J. Biol. Chem. 237*, 1982.

Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961) *J. Biol. Chem. 236*, 2277.

Richards, O. C., and Rutter, W. J. (1961), *J. Biol. Chem. 236*, 3177.

Rose, I. A. (1960), *J. Biol. Chem. 235*, 1170.

Sandell, E. B. (1959), Colormetric Determination of Traces of Metals, New York, N. Y., Interscience, p. 608.

Schwarzenbach, G. S. and Sulzberger, R. (1943), *Helv. Chim. Acta 26*, 453.

Suelter, C. H., and Melander, W. (1963), *J. Biol. Chem. 238*, PC4108.

Tietz, A., and Ochoa, S. (1958), *Arch. Biochem. Biophys. 78*, 477.
Von Korff, R. W. (1953), *J. Biol. Chem. 203*, 265.
Warner, R. C., (1958), *Arch. Biochem. Biophys. 78*, 494.
Weber, G. (1965), *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., eds., New York, N. Y., Academic (in press).

The Effect of Hydrogen Peroxide on Glucose Oxidase from *Aspergillus niger**

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ABSTRACT: The effect of H_2O_2 on the flavoenzyme glucose oxidase from *Aspergillus niger* has been studied at pH 5.8 and 25°. The enzyme is inactivated by H_2O_2. The reduced form is much more sensitive to H_2O_2 and is inactivated at least 100 times more readily than the oxidized form. The rate of inactivation of the reduced form is not dependent on the concentration of D-glucose. Properties of the inactive enzyme have also been studied. The molecular weight of the inactive glucose oxidase is the same as for the untreated enzyme, whereas the spectrum differs slightly. However, no chemical alterations were detected in the free flavin-adenine dinucleotide group after it had been released from the enzyme. Amino acid analysis showed that when oxidized and reduced glucose oxidase were treated with H_2O_2 under identical conditions slightly more methionine sulfoxide was found in the reduced H_2O_2-treated enzyme than in the oxidized H_2O_2-treated enzyme. It is therefore suggested that the inactivation of the enzyme involves modification of certain methionine residues located at or near the active site. Possible mechanisms for the inactivation of the reduced enzyme are discussed.

Hydrogen peroxide has recently received increased interest in the area of enzyme chemistry because of its ability to modify certain amino acid residues in proteins. It has been shown that at acid pH values methionine is easily oxidized to methionine sulfoxide by H_2O_2 and at basic pH values tryptophan is destroyed. Koshland *et al.* (1962) have studied the effect of H_2O_2 on chymotrypsin and have shown that it oxidizes a single methionine residue situated two residues away from the active serine. This change in the enzyme resulted in a threefold increase in K_m, whereas V_{max} remained the same. Kassel (1964) and Neumann *et al.* (1962) have studied the effect of H_2O_2 on the basic trypsin inhibitor and ribonuclease. In both cases it was shown that oxidation of methionine to methionine sulfoxide had occurred. Hachimori *et al.* (1964) have investigated the effect of H_2O_2 at alkaline pH values on several proteins and found that tryptophan is destroyed.

Within the field of flavoproteins there are a number of flavoenzymes whose natural hydrogen acceptor appears to be oxygen and which therefore will produce H_2O_2 under aerobic conditions in the presence of suitable substrates. However, little is known about the effect of H_2O_2 on these proteins. Bernheim and Dixon (1928) noted some activation of xanthine oxidase with very low concentrations of H_2O_2 and decreased activity with higher concentrations of H_2O_2.

The present study was undertaken in an attempt to gain a better understanding of the effect of H_2O_2 on one of these enzymes, glucose oxidase from *Aspergillus niger* (E.C. 1.1.3.4). This enzyme catalyzes the reaction

$$\beta\text{-D-glucose} + \text{E-FAD} \longrightarrow \text{E-FADH}_2{}^{1} + \delta\text{-D-gluconolactone} \quad (1)$$

$$\text{E-FADH}_2 + O_2 \longrightarrow \text{E-FAD} + H_2O_2 \quad (2)$$

where E-FAD stands for one active site. The enzyme contains two FAD groups per molecule. It has recently been purified and the properties of it studied in some detail (Gibson *et al.*, 1964; Pazur and Kleppe, 1964; Pazur *et al.*, 1963; Pazur *et al.*, 1965).

It appears from the present work that H_2O_2 severely affects the reactivity of this enzyme. The reduced form of the enzyme is particularly sensitive to H_2O_2 and is inactivated much more rapidly than the oxidized form.

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[1] Abbreviations used in this work: FAD, flavin-adenine dinucleotide; $FADH_2$, reduced FAD; *p*-MB, *p*-mercuribenzoic acid.