The Effect of Temperature on the Kinetics of Adenosine Diphosphoglucose Pyrophosphorylase from *Rhodospirillum rubrum**

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ABSTRACT: A study of the effect of temperature on the reaction catalyzed by adenosine diphosphoglucose pyrophosphorylase from *Rhodospirillum rubrum* has shown that, in the absence of the allosteric activator of the enzyme, pyruvate, a plot of log V_{max} *vs.* $1/T$ deviates from linearity at temperatures above 26°. The curve shows a continuous decrease in slope up to 50°, finally reaching an initial velocity of 10% the maximal rate, which is found at 35°. However, in the presence of pyruvate the curve is linear up to 32° and the initial velocity continually increases with increasing temperature up to 55°. The inactivation at elevated temperature has been shown to be freely reversible. Above 60° the enzyme undergoes irreversible denaturation. The linear portions of the Arrhenius plots give values for the enthalpy of activation of the synthesis reaction of 11.2 kcal/mole in the presence of pyruvate and 14.2 kcal/mole in its absence. The temperature dependence of the equilibrium constant gives a value of 735 cal/mole for the standard enthalpy change of the synthesis reaction. Preliminary studies of the effect of temperature on the reverse (pyrophosphorolysis) reaction gave similar nonlinear Arrhenius plots. As the temperature of incubation was decreased, the concentration of substrates and divalent metal ion needed for half-maximal velocity ($S_{0.5}$ and K_m) were found to decrease, as did the Hill interaction coefficients for ATP and $MgCl_2$. The combined results could be interpreted in terms of a three-state model, one high-temperature, inactive form and two low-temperature, active forms of the enzyme with the low-temperature forms stabilized by pyruvate. Thermodynamic parameters were calculated for the equilibria between the forms and the values obtained were consistent with those expected for a temperature-dependent conformational change of the enzyme.

A study of the effect of temperature on the kinetic properties of a number of enzymes has shown that they do not conform to that expected from the Arrhenius equation, *i.e.*, a linear relationship between the logarithm of the velocity and the reciprocal of the absolute temperature (see Massey *et al.*, 1966, for a partial list). Dixon and Webb (1964) have discussed nonlinear Arrhenius plots with emphasis on the factors which can cause this anomalous behavior. Of the numerous enzymes which exhibit nonlinear Arrhenius profiles, the best studied examples (Massey *et al.*, 1966; Suelter and Melander, 1963; Kayne and Suelter, 1965, 1968; Suelter *et al.*, 1966; Suelter, 1967) appear to have one thing in common: the enzyme can exist in two or more stable conformational forms over different temperature ranges and these forms possess different kinetic properties. In this paper we present kinetic results for ADP-glucose pyrophosphorylase of *R. rubrum* which can be interpreted in this way. The possible relationship between these results and the allosteric properties of the enzyme are discussed.

Materials and Methods

Reagents. ATP[1] (Calbiochem) solutions were adjusted to pH 5–6 and their concentrations were determined by their absorbance at 259 nm (ϵ 1.54 × 10^4 l. $mole^{-1}$ cm^{-1}). ADP-glucose (Calbiochem, A grade) was assayed by its absorbance at 259 nm (ϵ 1.54 × 10^4 l. $mole^{-1}$ cm^{-1}) and NADH (P.L. Biochemicals) and NADPH (Sigma) were assayed spectrally at both 340 and 260 nm. Glucose 1-phosphate (Calbiochem) and [^{14}C]α-glucose 1-phosphate (Amersham Searle Corp.) were assayed with phosphoglucomutase (Sigma), glucose 6-phosphate dehydrogenase (Boehringer-Mannheim), and NADPH (Bergmeyer, 1963a). [^{32}P]Pyrophosphate (New England Nuclear) was assayed for total, acid-labile, and inorganic phosphate by the method of Fiske and Subbarow (1925). Pyruvate (Sigma) was assayed with lactate dehydrogenase (Sigma) and NADH (Bergmeyer, 1963b). Bicine (*N*,*N*-bis(2-hydroxyethyl)glycine) and bovine plasma albumin (crystalline, A grade) were obtained from Calbiochem. All other chemicals were obtained from commercial sources.

Growth and Maintenance of R. rubrum. Stock cultures of *Rhodospirillum rubrum* were maintained in stab culture as described by Cohen-Bazire *et al.* (1957). Cultures were grown photosynthetically under semianaerobic conditions in the medium of Evans (1965) as described by Furlong and Preiss (1969).

Enzyme Assays

Assay A (Pyrophosphorolysis). The purification was monitored by measuring ADP-glucose pyrophosphorolysis in the presence of the activator, pyruvate, by following the formation of [^{32}P]ATP from ADP-glucose and [^{32}P]PP_i as described by Furlong and Preiss (1969).

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[1] Abbreviations used are: ADP-glucose, ADPG, adenosine diphosphoglucose; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Glc-1-P, glucose 1-phosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

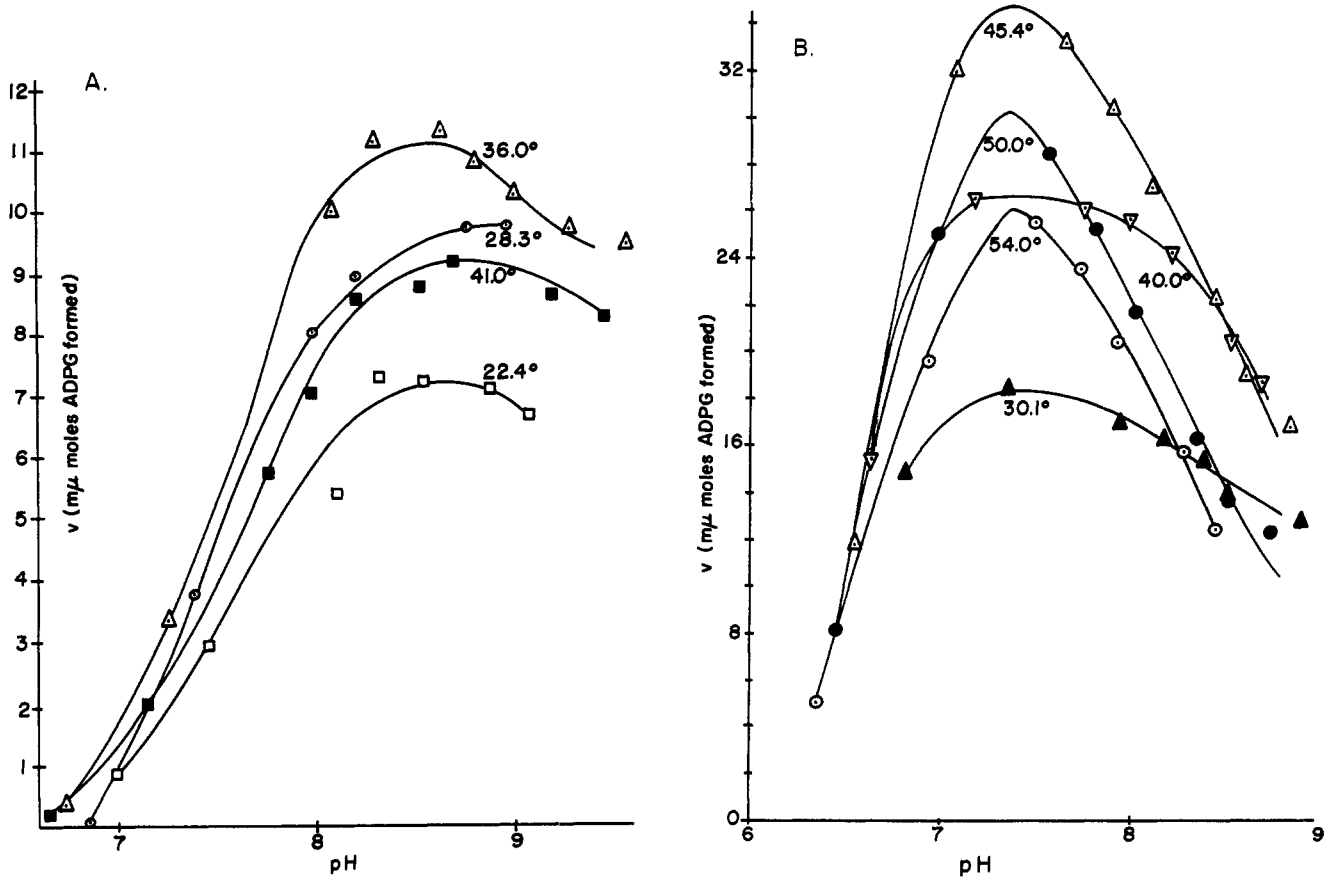


FIGURE 1: (A) pH dependence of ADP-glucose synthesis rate in the absence of pyruvate at several temperatures. The reaction mixture consisted of 20 μmoles of Bicine buffer, pH 8.6 (except when the pH was varied), 50 μg of crystalline bovine plasma albumin, 0.24 μmole of [^{14}C]glucose 1-phosphate, 1.5 μmoles of ATP, 2.2 μmoles of $MgCl_2$, and enzyme in a final volume of 0.20 ml. (B) pH dependence of ADP-glucose synthesis rate in the presence of pyruvate at several temperatures. The reaction mixture consisted of 20 μmoles of Bicine buffer, pH 7.5 (except when the pH was varied), 50 μg of crystalline bovine plasma albumin, 0.24 μmole of [^{14}C]-glucose 1-phosphate, 0.75 μmole of ATP, 1.4 μmoles of $MgCl_2$, 4.0 μmoles of PVA, and enzyme in a final volume of 0.20 ml.

Assay B (*Unactivated Synthesis*). The synthesis of ADP-glucose was measured by following the formation of ADP-[^{14}C]glucose from ATP and [^{14}C]glucose-1-P as described by Ghosh and Preiss (1966). The reaction mixture contained 20 μmoles of Bicine buffer (adjusted to be pH 8.6 at the incubation temperature), 50 μg of crystalline bovine plasma albumin, [^{14}C]glucose-1-P, ATP, and $MgCl_2$ in the amounts shown in the figure legends, and enzyme in a final volume of 0.20 ml. The reactions mixtures were incubated for time intervals which gave linearity at the given temperature; the reaction was terminated by heating for 1 min in a boiling water bath and then assayed for ADP-[^{14}C]glucose. Data were corrected for a 10-min incubation (see treatment of data).

Assay C (*Activated Synthesis*). The conditions of this assay are the same as in assay B except the Bicine buffer was adjusted to pH 7.5 at the incubation temperature and 4.0 μmoles of pyruvate was added.

Assay D (*Determination of* K_{eq}). The equilibrium constant was determined by enzymatically following the changes in the concentrations of ATP and glucose 1-phosphate as equilibrium was approached from the forward (synthesis) and from the reverse (pyrophosphorolysis) direction as described by Paule and Preiss (1971).

Purification of ADP-glucose Pyrophosphorylase. The enzyme was purified approximately 530-fold using the procedure of Furlong and Preiss (1969) with the following modifications. After the ammonium sulfate fractionation step (step 4), 95 ml (950 mg; specific activity 28.4 μmoles of ATP formed/mg per 10 min) of the dialyzed ammonium sulfate fraction was adsorbed on a 2.4 × 35 cm DEAE-cellulose column previously equilibrated with 0.02 M potassium phosphate buffer (pH 7.0). The column was washed with a resin bed volume of 0.02 M potassium phosphate buffer (pH 7.0) and the enzyme was then eluted with a linear gradient which consisted of 1700 ml of 0.02 M potassium phosphate buffer (pH 7.0) in the mixing chamber and 1700 ml of 0.01 M potassium phosphate buffer (pH 7.0) containing 0.4 M KCl in the reservoir chamber. Enzymatic activity was located using the spectral assay (assay E, ref 13). The peak fractions were pooled and concentrated by bringing the eluate to 82% saturation with solid ammonium sulfate. After 15 min the solution was centrifuged for 15 min at 13,000*g*. The supernatant solution was decanted, and the precipitate was resuspended in a minimum volume of 0.05 M Hepes buffer (pH 7.0) and dialyzed overnight against the same buffer.

A column containing DEAE-Sephadex (2.5 × 25 cm) was equilibrated with 0.02 M phosphate buffer (pH 7.5), and the protein in 20 ml of the DEAE-cellulose dialysate (8.0 mg/ml, specific activity 97.5 μmoles/mg per 10 min) was adsorbed to the column. After washing the column with a resin bed volume of 0.02 M potassium phosphate buffer (pH 7.5), the enzyme was eluted with a linear gradient consisting of 1 l. of 0.02 M potas-

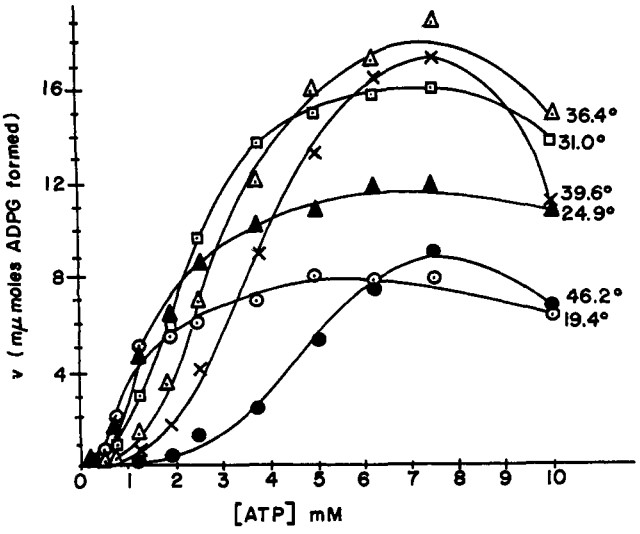


FIGURE 2: Dependence of ADP-glucose synthesis rate on ATP concentration in the absence of pyruvate at several temperatures. Reaction mixtures were identical with those in Figure 1A except the ATP concentration was varied.

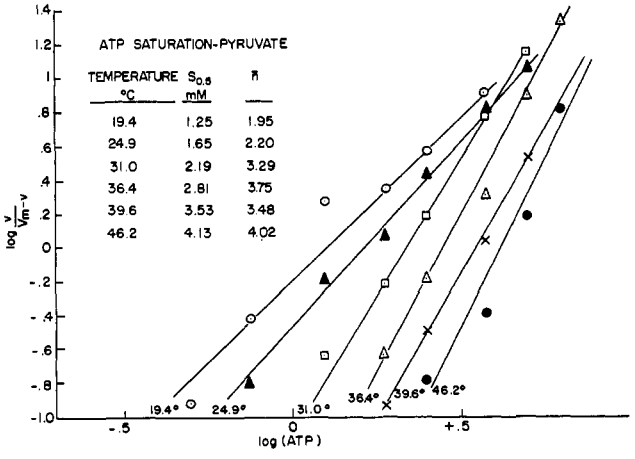


FIGURE 3: The data from Figure 2 plotted as log $[v/(V_{max} - v)]$ *vs.* the log of the ATP concentration (Hill plot).

sium phosphate buffer (pH 7.5) in the mixing chamber and 1 l. of 0.1 M phosphate buffer (pH 7.0) containing 0.3 M KCl in the reservoir chamber. The active fractions were located and concentrated as described above.

The concentrate (10.4 ml, 2.64 mg/ml, specific activity 250 μmoles of ATP formed/mg per 10 min) from the DEAE-Sephadex column was "dialyzed" against 1% glycine by repeated concentration and dilution using a Diaflo ultrafiltration cell manufactured by the Amicon Corporation (Cambridge, Mass.) with an XM-50 membrane. The sample was then electrofocused in a pH 3 to 10 gradient using an LKB 8102 electrofocusing column manufactured by LKB Instruments, Inc., Rockville, Md. The technique is described by Haglund (1967). The sample was added after approximately one-third of the gradient had been layered into the column. The cathode was placed at the top of the column. After 40 hr, the column was drained and the active fractions were located using the spectral assay. The peak fractions, at pH 5.0 to 5.2, were pooled, adjusted to pH 6.95 with 1.0 M Hepes (pH 7.5), and dialyzed against several changes of 0.05 M Hepes (pH 7.0) for 12 hr. The dialysate was then concentrated in an Amicon ultrafiltrator and any remaining sucrose removed by repeated dilution and concentration. The resulting preparation was used as the enzyme source (1.06 mg/ml, specific activity 363 μmoles of ATP formed/mg per 10 min). The overall yield was approximately 10%.

Treatment of Data. All kinetic measurements were run at two protein concentrations and the velocity of the reactions were always linear with protein. Raw data from the synthesis assays (assays b and c) were corrected for losses or product from the DEAE paper disks during the washing procedure.

Results

Effect of Temperature

Linearity of Assay with Time. In the absence of pyruvate, the formation of product with time is linear for at least 10 min at 46°, the highest temperature used in studying the unactivated reaction.

In the presence of pyruvate the assay is linear for at least 10 min at 50° and for at least 2.5 min at 55°. These results indicate that by shortening the length of the normal 10-min incubation time at very high temperatures, the rate of the reaction can be measured without interference due to instability of the enzyme. Linearity with protein was checked in each subsequent experiment by using two protein concentrations for each data point.

pH Optimum as a Function of Temperature. Nonlinear Arrhenius plots can be due to an effect of temperature on the pH functions of any or all of the components of the reaction mixture (Dixon and Webb, 1964). In order to eliminate this effect as a possible cause of the anomalous plot obtained for this enzyme, the pH optimum for the reaction was determined at several temperatures. The pH profiles of the synthesis reaction in the absence (Figure 1A) and in the presence (Figure 1B) of pyruvate have been determined as a function of temperature. Although the pH optima for the activated (7.4) and the unactivated (8.6) reactions differ by more than one pH unit (Furlong and Preiss, 1969), the optimum for each does not vary significantly with changes in incubation temperature. It should be pointed out that the pK_a of Bicine buffer varies considerably with temperature (Good *et al.*, 1966). Thus, it was necessary to prepare separate buffer solutions for each incubation temperature used in this study, each titrated to be the proper pH at the incubation temperature.

Another possible explanation for nonlinear Arrhenius plots is a change in the affinity of the enzyme for one or more of its substrates (Dixon and Webb, 1964). If the heat of binding of a substrate to the enzyme is large, the affinity of the enzyme for that substrate will change significantly with temperature. Thus, even if the enzyme is saturated with that substrate at one temperature, the use of the same "saturating" concentration over the entire temperature range does not ensure saturating conditions at all temperatures. Since the rate of the reaction measured at a nonsaturating temperature will be less than the actual maximum velocity, the resulting Arrhenius plot will be concave downward. Although the direction of the error introduced into the value of energy of activation measured over a narrow temperature range depends on whether the binding of substrate is endo- or exothermic, the shape of the curve is always concave downward. This problem has been discussed by Gibson (1953). The effect of temperature on the affinity of ADP-glucose pyrophosphorylase for its substrates was therefore examined.

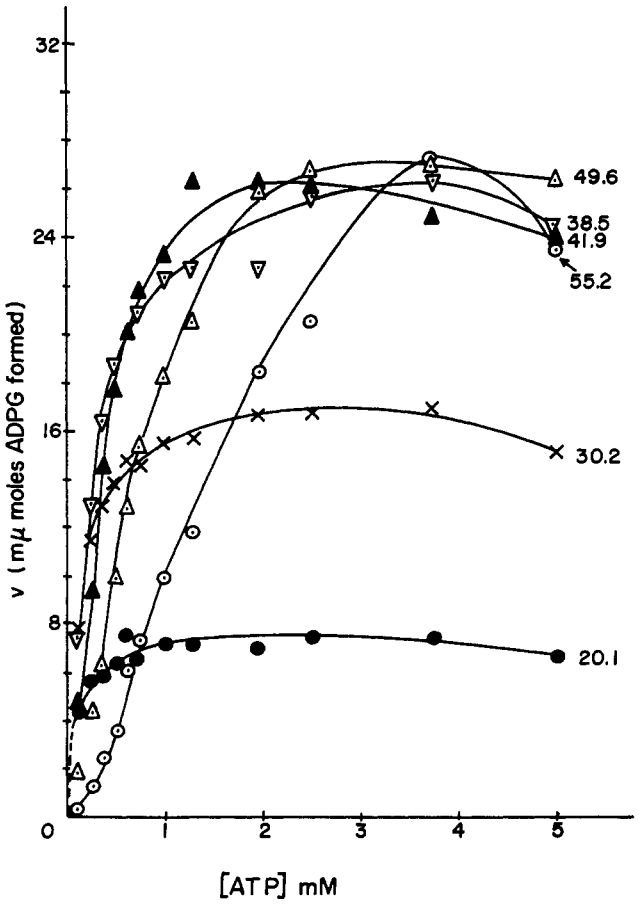


FIGURE 4: Dependence of ADP-glucose synthesis rate on ATP concentration in the presence of pyruvate at several temperatures. The reaction mixtures were identical with those in Figure 1B except the ATP concentration was varied.

Effect of Temperature on the ATP Saturation Curves. Figure 2 shows the effect of temperature on the ATP saturation curve in the absence of pyruvate. The concentration of ATP needed for half-maximal velocity decreases as the temperature of incubation is lowered. However, the same concentration of ATP (7.5 mM) may be used to obtain maximal velocity at all of the temperatures studied.

Figure 3 shows the data of Figure 2 plotted as log $[v/(V_{max} - v)]$ *vs.* log ATP concentration (Hill plot).

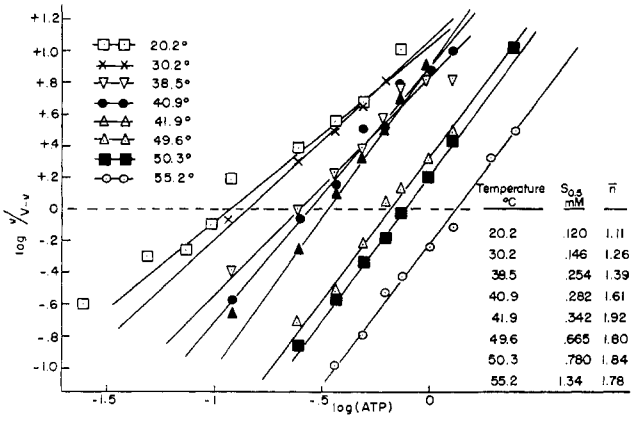


FIGURE 5: Hill plot of data from Figure 4.

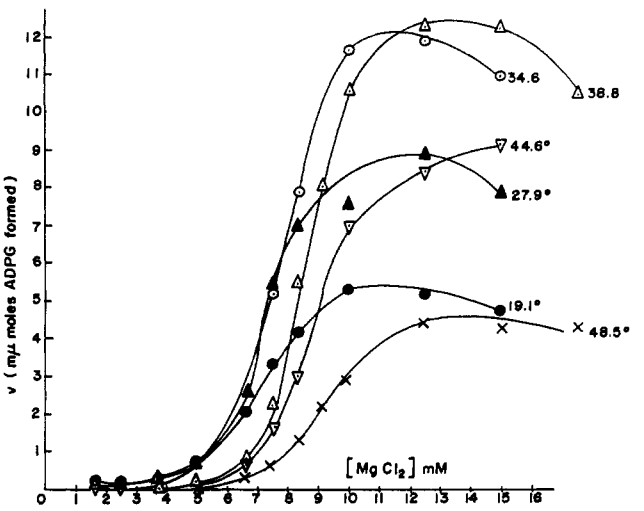


FIGURE 6: Dependence of ADP-glucose synthesis rate on $MgCl_2$ concentration in the absence of pyruvate at several temperatures. The reaction mixtures were identical with those in Figure 1A except the $MgCl_2$ concentration was varied.

The $S_{0.5}$ values and the $\bar{n}$ values taken from this plot are tabulated in the figure. The $S_{0.5}$ values decrease from 4 to 1.25 mM as the temperature is decreased from 46 to 19° and the interaction coefficient decreases from 4 to 2 over the same temperature range.

Figure 4 shows the effect of temperature on the ATP saturation curves in the presence of the activator, pyruvate, and Figure 5 shows Hill plots of these data. Although the concentration of ATP needed for half-maximal velocity at any given temperature is decreased in the presence of pyruvate as demonstrated by Furlong and Preiss (1969), the effect of temperature is qualitatively the same as in the unactivated case. The $S_{0.5}$ for ATP decreases as the temperature is decreased, but one concentration (3.75 mM) will yield maximal velocity at all temperatures tested. The $S_{0.5}$ and $\bar{n}$ values for ATP at several

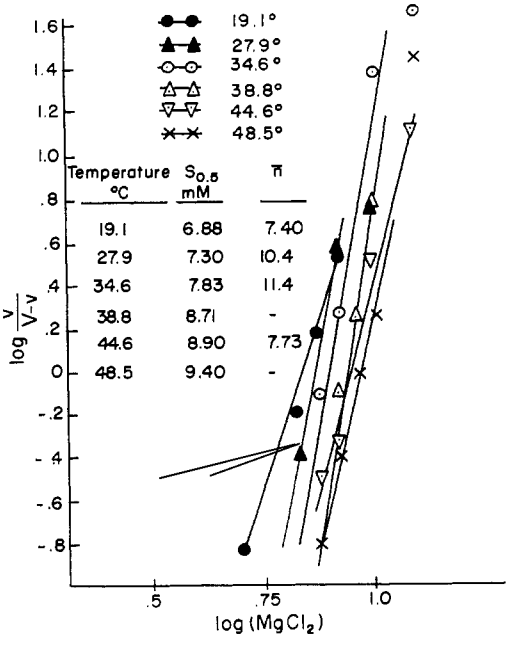


FIGURE 7: Hill plot of data from Figure 6.

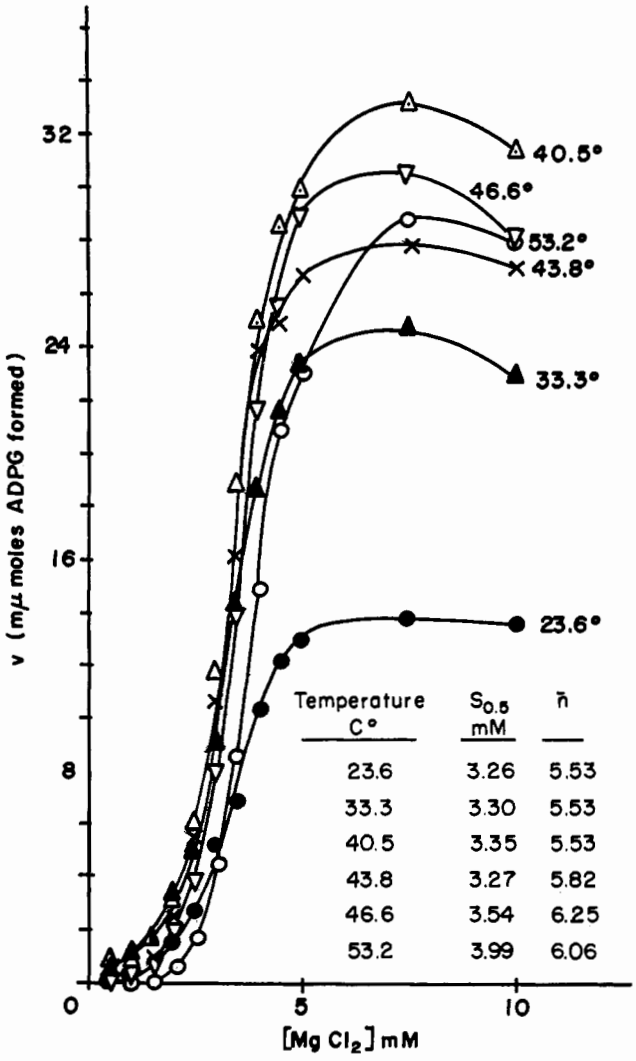


FIGURE 8: Dependence of ADP-glucose synthesis rate on $MgCl_2$ concentration in the presence of pyruvate at several temperatures. The reaction mixtures were identical with those in Figure 1B except the concentration of $MgCl_2$ was varied.

temperatures are tabulated in Figure 5. The $S_{0.5}$ values decrease from 1.3 mM at 55° to 0.12 mM at 20°. The interaction coefficient decreases from 1.78 at 55° to 1.1 at 20°. The saturation curve for ATP is, therefore, essentially hyperbolic at 20°.

Effect of Temperature on $MgCl_2$ Saturation Curves. The effect of temperature on the $MgCl_2$ saturation curves in the absence of pyruvate is shown in Figure 6. Hill plots of these data are shown in Figure 7 and the $S_{0.5}$ and $\bar{n}$ values at several temperatures are shown in the figure. The $S_{0.5}$ values decrease slightly with decreasing temperature (from 9.4 mM at 48° to 6.9 mM at 19°). The concentration of $MgCl_2$ needed for saturation is approximately the same at all temperatures tested (11 mM) and is only slightly higher than the $S_{0.5}$ values due to the extremely large interaction coefficients exhibited by this ligand.

$MgCl_2$ saturation curves at several temperatures in the presence of pyruvate are shown in Figure 8. The $S_{0.5}$ and $\bar{n}$ values at several temperatures are tabulated in Figure 8. The $S_{0.5}$ values vary only slightly with temperature (from 4 mM at 53° to 3.26 mM at 24°), but are significantly lower than in the absence of pyruvate at any given temperature. The $\bar{n}$ values are also smaller than in the absence of pyruvate (6.1 at 53°, 5.5 at 24°). One concentration (7 mM) may be used for saturation at all temperatures tested.

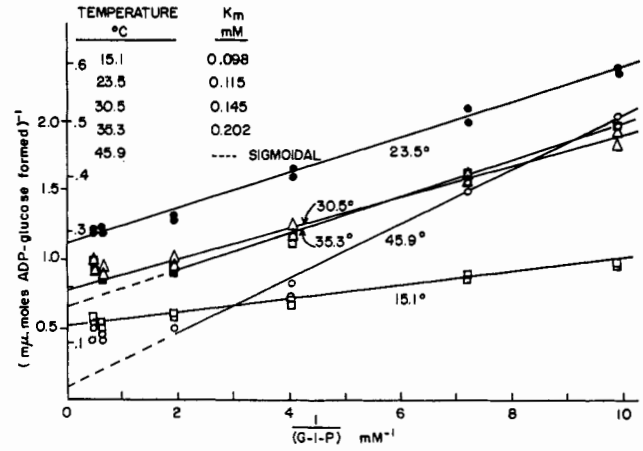


FIGURE 9: Dependence of ADP-glucose synthesis rate on the glucose 1-phosphate concentration in the absence of pyruvate at several temperatures plotted as the reciprocal of the rate of ADP-glucose formation *vs.* the reciprocal of the glucose 1-phosphate concentration. The reaction mixtures were identical with those in Figure 1A except the glucose 1-phosphate concentration was varied. The 0–2.0 scale on the ordinate was used for the 45.9° curve only.

Effect of Temperature on Glucose 1-Phosphate Saturation Curves. The dependence of the rate of the reaction on glucose 1-phosphate as a function of different incubation temperatures in the absence of pyruvate is shown in Figure 9. At low temperatures the curves are hyperbolic whereas at higher temperatures (45.9°) they appear to be slightly sigmoidal. The figure shows a slight decrease in K_m values obtained as the temperature is decreased (from approximately 0.20 mM at 35° to 0.098 mM at 15°).

Figure 10 shows the dependence of the reaction rate on glucose 1-phosphate in the presence of pyruvate. From 17 to 51° the curves are hyperbolic, and the variation of K_m with tem-

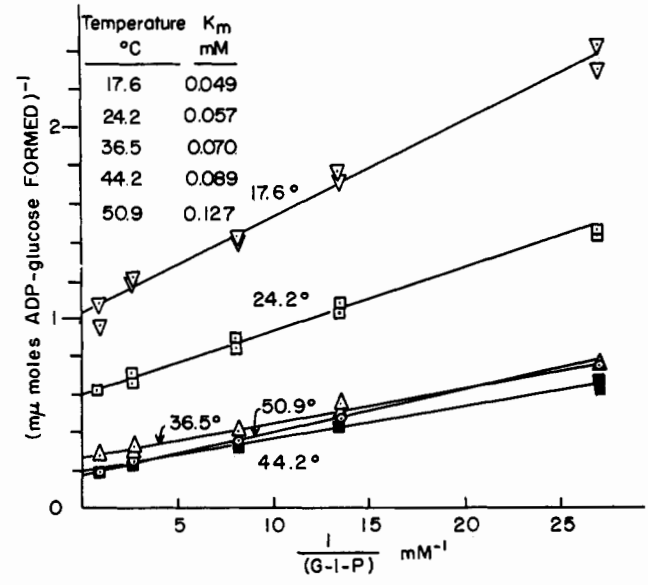


FIGURE 10: Dependence of the rate of ADP-glucose formation on glucose 1-phosphate concentration in the presence of pyruvate at several temperatures plotted as the reciprocal of the rate of ADP-glucose formation *vs.* the reciprocal of the glucose 1-phosphate concentration. The reaction mixtures were identical with those in Figure 1B except the glucose 1-phosphate concentration was varied.

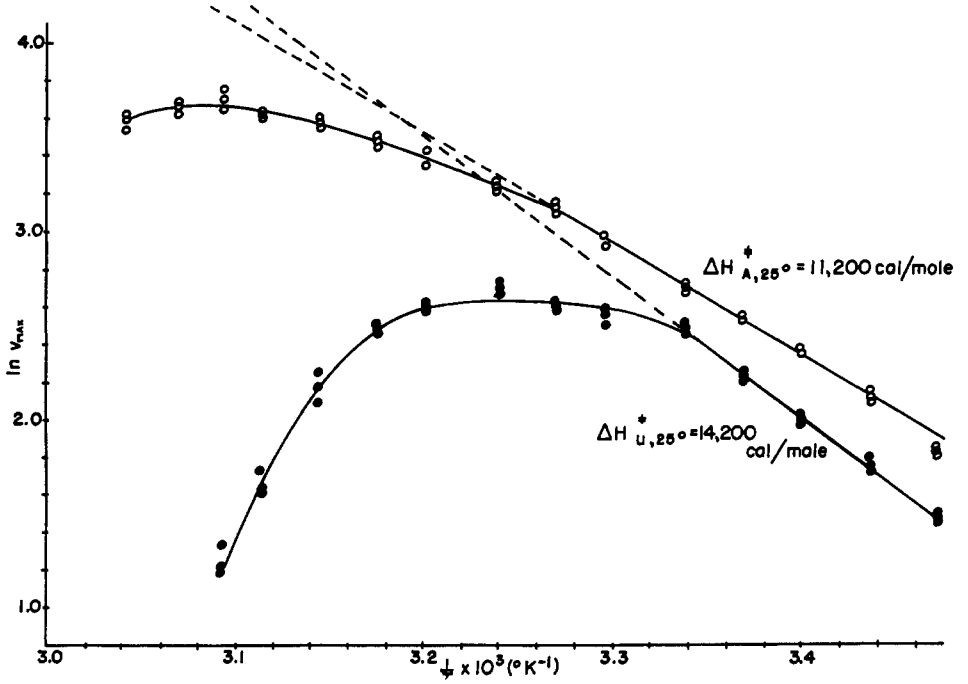


FIGURE 11: Plot of the natural log of the rate of ADP-glucose synthesis *vs.* the reciprocal of the absolute temperature. The upper curve (open circles) is in the presence of pyruvate and reaction mixtures are identical with those in Figure 1B. The lower curve (closed circles) is in the absence of PVA and reaction mixtures are identical with those in Figure 1A.

perature (Figure 10) indicates a decreased K_m for glucose 1-phosphate at lower temperatures.

For both the activated and the unactivated reaction, 1.2 mM glucose 1-phosphate is sufficient to saturate the enzyme over the temperature range studied.

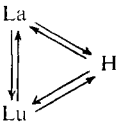
Effect of Temperature on the Maximum Velocity of the Synthesis Reaction. Figure 11 shows the effect of temperature on the rate of the reaction under the optimal conditions for the activated and the unactivated reaction as described in the previous section.

In the absence of pyruvate (lower curve) the plot deviates from the expected linearity at temperatures above 26° and has an enthalpy of activation (ΔH^*) of 14,200 cal/mole below this temperature. The activated reaction, however, is linear up to 32° and does not deviate from the expected linear behavior as drastically as the unactivated reaction. The activated reaction exhibits a ΔH^* of 11,200 cal/mole below 32°.

If the enzyme is assayed under the optimal conditions for the activated reaction except at the pH optimum of the unactivated reaction (*i.e.*, pH 8.6, 3.75 mM ATP, 1.2 mM glucose 1-phosphate, 7 mM $MgCl_2$, 20 mM pyruvate), the general shape of the resulting curve is almost identical with that found at the pH optimum for the activated reaction. The temperature at which deviation from linearity first appears is approximately the same, and the ΔH^* is almost identical. The differences between the activated and unactivated Arrhenius plots are, therefore, not due solely to the different hydrogen ion concentrations in the two reaction mixtures.

Preliminary studies of the effect of temperature on the reverse (pyrophosphorolysis) reaction indicate similar behavior. In the presence or in the absence of pyruvate, the curves deviate from linearity. As in the synthesis direction, pyruvate has a marked effect on the shape of the curve. Not only is the temperature of the "break" in the curve raised by the presence of pyruvate, but the magnitude of the deviation from linearity is markedly reduced.

Determination of Thermodynamic Parameters for a High-Temperature–Low-Temperature Equilibrium. The results can be interpreted in terms of a simple model in which the enzyme is capable of existing in three conformational forms, one high (H) and two low-temperature forms (La and Lu), with only the low-temperature forms exhibiting catalytic activity.



(active forms) (inactive form)

One of the low-temperature forms is designated the "activated" form (La). It has a pH optimum of 7.4 and an enthalpy of activation of 11.2 kcal/mole. The other low-temperature form, the "unactivated" form (Lu), differs from the activated form in its pH optimum of 8.6 and its enthalpy of activation of 14.2 kcal/mole. According to the model, in the presence of pyruvate the La form predominates over the Lu form. Pyruvate also stabilizes the active forms of the enzyme over the inactive (H) form.

Using this model, the equilibrium constant between the high- and the low-temperature forms of the enzyme can be calculated as a function of temperature in the following manner. The amount by which the observed rate (V_{obsd}) differs from the expected rate (V_{ex}), obtained by extrapolation of the linear portion of the Arrhenius plot, is assumed to be directly proportional to the fraction of the enzyme in the high-temperature form. The observed rate is therefore directly proportional to the fraction of the enzyme in the low-temperature

TABLE I: Reversibility of Temperature Effect.

Tube	Length of Incubation 30°	Length of Incubation 47°	mμmoles of ADP-glucose Formed
1	5 min		3.03[a]
2		5 min	1.38
3	5 min →	5 min	4.39
4	5 min ←	5 min	4.67

[a] Sum of tubes 1 and 2 = 4.41 mμmoles.

form. The equilibrium constant at any temperature for the H to La or the H to Lu equilibrium, depending upon the presence or absence of pyruvate, respectively, is given by

$$K_{eq}(T) = \frac{\text{fraction in high-temperature form } (T)}{\text{fraction in low-temperature form } (T)} = \frac{V_{ex}(T) - V_{obsd}(T)}{V_{obsd}(T)}$$

where $K_{eq}(T)$, $V_{ex}(T)$, and $V_{obsd}(T)$ are the parameters described above at any given temperature. This method has been used to calculate a similar equilibrium constant for native and denatured RNase (Kalnitsky and Resnick, 1959). Calculation of $K_{eq}(T)$ at several temperatures from the data of Figure 11 and plotting ln $K_{eq}(T)$ *vs.* $1/T$ yield the curves shown in Figure 12. The slope of this plot is equal to $-\Delta H°/R$, where $\Delta H°$ is the standard enthalpy change for the proposed interconversion of enzyme forms.

Figure 12 shows that $\Delta H°$ is a constant over the temperature range of interest, and calculation of $\Delta S°(T)$ at several temperatures shows that it is also a constant in this temperature range. In the absence of pyruvate (upper curve), the values for $\Delta H°$ and $\Delta S°$ are 45,800 cal/mole and 147.6 eu, respectively. In the presence of the activator (lower curve), $\Delta H°$ = 28,500 cal/mole and $\Delta S°$ = 86.4 eu.

Reversibility of the Temperature Effect. In order to show that the inactivation at elevated temperature is a rapidly reversible phenomenon, four identical reaction mixtures were incubated in the following manner: (1) 5 min at 30° (approximately the optimal temperature for the unactivated reaction), (2) 5 min at 47° (a temperature which gives approximately half the rate observed at 30°), (3) 5 min at 30° and then quickly transferred to a 47° bath for an additional 5 min, and (4) 5 min at 47° and then an additional 5 min at 30°. If the decreased catalytic activity observed at the higher temperature is due either to an irreversible process or to a slowly reversible process, the rate observed in tube 4 would be less than the sum of the rates of tubes 1 and 2. If the transition from the low-temperature active forms to the high-temperature inactive form was a slow process, the sum of the rates of tubes 1 and 2 would be less than the rate of tube 3. If the transition in both directions was rapidly reversible, the sum of the rates of tubes 1 and 2 would be equal to the rates of tubes 3 and 4. The results of such an experiment are shown in Table I. The results indicate that the transition is reversible and is so rapid that its rate cannot be measured by this method.

Determination of $\Delta H°$ for the Synthesis Reaction. The equilibrium constant for the forward (synthesis) reaction was determined as a function of temperature as described in the Methods section. The data were plotted according to the van't Hoff equation as described above. The plot is shown in Figure 13. The slope of this plot yields a value of 735 cal/mole for the standard enthalpy of the forward reaction.

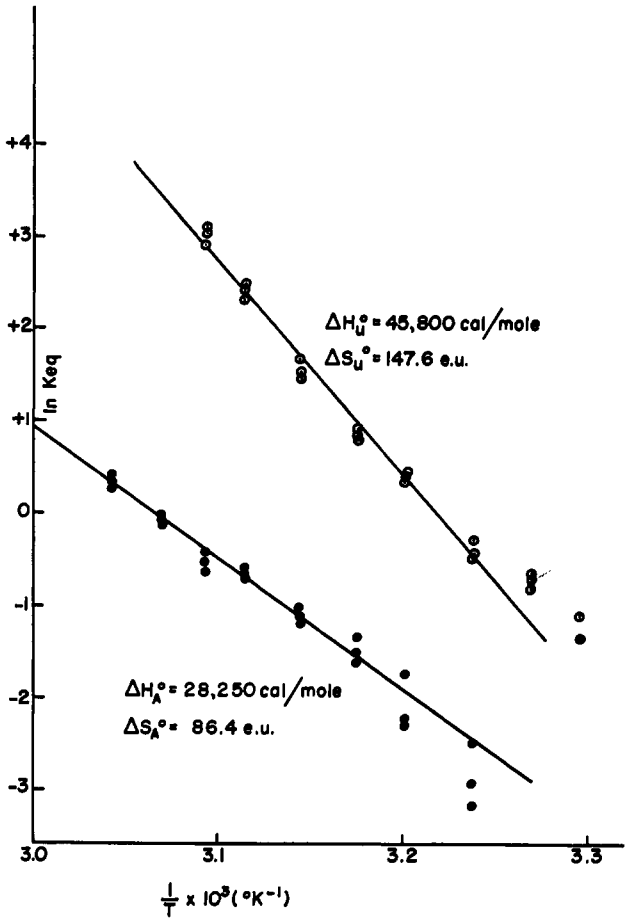


FIGURE 12: Plot of the natural log of the equilibrium constant for the high-temperature to low-temperature transition *vs.* the reciprocal of the absolute temperature. The upper curve (open circles) is for the reaction in the absence of pyruvate and the lower curve (closed circles) is for the equilibrium in the presence of pyruvate. Details on calculation of the equilibrium constant are given in the text.

Discussion

Pyruvate is known to be an allosteric effector of ADP-glucose pyrophosphorylase from *R. rubrum*. It affects the maximal velocity, concentration of ATP, $MgCl_2$, and glucose 1-phosphate needed for half-maximal velocity and changes the pH optimum of the enzymatic reaction (Furlong and Preiss, 1969). Allosteric effectors are thought to exert their effect by altering the conformation of the enzyme (Monod *et al.*, 1965; Koshland *et al.*, 1966).

As shown in this study, pyruvate also has an effect on the anomaly observed in the temperature profile of this enzyme. We hypothesize, therefore, that the temperature phenomenon is due to a temperature-dependent conformational change of the enzyme which is effected by pyruvate. The thermodynamic quantities determined in this study are of the proper magnitude for such a conformational change (Massey *et al.*, 1966; Kalnitsky and Resnick, 1959), and similar temperature-dependent conformational changes have been observed in other

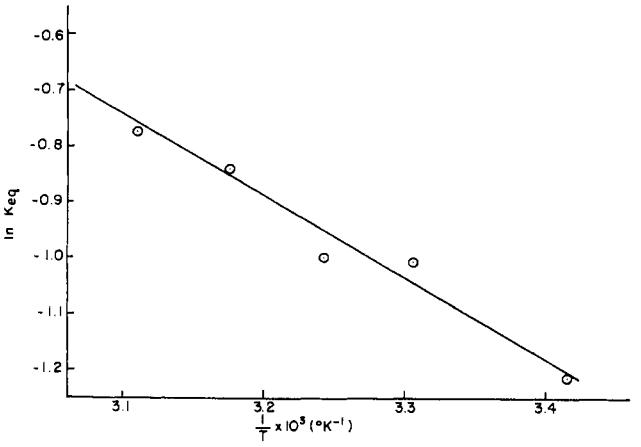


FIGURE 13: Plot of the natural logarithm of the equilibrium constant for the synthesis reaction *vs.* the reciprocal of the absolute temperature. Details of the method used to determine the equilibrium constant are described by Paule and Preiss (1971).

systems. Since the phenomenon has been observed for both the forward and the reverse reaction, with two different sets of substrates and two entirely different assay procedures, it appears to be very unlikely that the effect can be on any component of the system other than the enzyme itself. Studies of the effect of temperature on the concentrations of substrate and divalent metal ion needed for maximal velocity indicate that changes in the concentration of substrate needed to achieve maximal velocity cannot account for the anomalous Arrhenius plots. The linear production of product as a function of time at elevated temperature and the rapid reversibility of the temperature effect allow rejection of irreversible denaturation of the enzyme as a possible explanation. The pH optimum is a constant over the temperature range of interest, and the same "stabilizing" effect of pyruvate is observed at two different hydrogen ion concentrations. Therefore, the phenomenon is probably not due to changes in the degree of ionization of groups on the enzyme.

The results are compatible with the model presented under Results, consisting of three rapidly interconvertible conformers, one high-temperature inactive form (H) and two low-temperature active forms (La and Lu).

The similarities between the allosteric effects of pyruvate and the effects of lowered temperature of incubation deserve mention. The addition of pyruvate decreases the concentration needed for half-maximal velocity of the ligands ATP, $MgCl_2$, and glucose 1-phosphate. Pyruvate also decreases the Hill interaction coefficient for those substrates which exhibit sigmoidal saturation curves (including glucose 1-phosphate at elevated temperature which had previously been thought to exhibit hyperbolic saturation curves only). Furlong and Preiss (1969) have shown that a reciprocal heterotropic interaction exists between ATP and pyruvate. Increasing the concentration of either ligand causes a corresponding decrease in the homotropic interaction observed for the alternate ligand as mirrored by a decrease in the Hill interaction coefficient.

Lowering the incubation temperature also decreases the concentration of substrate or divalent metal ion needed for half-maximal velocity and decreases the homotropic interaction observed for ATP, $MgCl_2$, and glucose 1-phosphate. ATP and pyruvate exhibit a reciprocal heterotropic interaction, which implies that they have similar effects on the conformation of the enzyme. A logical extension of this is that ATP as well as pyruvate may stabilize the active, low-temperature forms of the enzyme. This effect does not readily lend itself to kinetic analysis, however. It is very difficult to distinguish between a stabilizing effect and an effect due to decreasing the ATP concentration below a value which is saturating over the entire temperature range since both lead to the same type of anomalous Arrhenius plots. A study of the physical properties of the enzyme as a function of temperature and ATP and pyruvate concentration should clarify this point. In addition, a study of this type might supply general information previously not available about allosteric enzymes. Although several allosteric enzymes are known to exhibit "temperature-sensitive" transitions, none have been studied in enough detail to rule out causes for this phenomenon other than the existence of several conformational forms of the enzyme.

It should be noted that pyruvate has at least two effects on the enzyme which are not mimicked by either of the other conditions, high ATP or low temperature. In the presence of pyruvate the pH optimum is shifted from 8.6 to 7.4 and the enthalpy of activation is lowered by 3 kcal/mole. These effects are not observed even at low temperature in the presence of saturating ATP. Therefore, pyruvate must have some effect on the conformation of the enzyme in addition to that involved in the reciprocal heterotropic interaction between ATP and pyruvate. It may be that the La form of the enzyme exists only in the presence of pyruvate and there may be other forms of the enzyme dependent on the degree of saturation of substrate and activator sites. Furlong and Preiss (1969) have studied the properties of this enzyme at 37°, a nonphysiological temperature for *R. rubrum* which will not grow at temperatures above 30°. They found that, unlike ADP-glucose pyrophosphorylase from a number of other sources, the *R. rubrum* enzyme is not inhibited by either ADP, AMP, or inorganic phosphate. The knowledge that lowered temperature greatly decreases the concentration of ligands needed by the enzyme to achieve half-maximal velocity led to a further examination of this lack of a potent inhibitor whose concentration would vary in response to the metabolic state of the cell. However, it was found that even at lowered temperatures none of the above compounds effectively inhibit the enzyme (E. Greenberg and J. Preiss, personal communication).

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Studies on Adrenal Steroid Hydroxylases. Reactivity of Iron Atoms in Adrenal Iron–Sulfur Protein (Adrenodoxin) with Iron-Chelating Agents*

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ABSTRACT: Kinetic studies on the reaction of adrenodoxin with iron chelators have been carried out in order to elucidate the reaction mechanism. The reaction rate of adrenodoxin with excess *o*-phenanthroline apparently follows zero-order kinetics under aerobic conditions, whereas it is a pseudo-first-order reaction under anaerobic conditions. The activation energies are 3.3, and 12.6 kcal per mole under aerobic and anaerobic conditions, respectively. Model experiments with inorganic compounds showed that the reaction of dithionite with Fe^{3+}–*o*-phenanthroline occurs about 400 times faster than with free Fe^{3+} ions, and that the reaction of *o*-phenanthroline with Fe^{2+} is very fast. In the presence of 4 M urea and 1 M KCl, the iron chromophore of adrenodoxin is quite stable, and the rate is faster than that of free Fe^{3+} ions at 26°. From these results, the reaction sequence is proposed to consist of at least binding of *o*-phenanthroline to the protein and subsequent reduction of Fe^{3+}–*o*-phenanthroline to its Fe^{2+} complex by the intramolecular reductants. The former reaction is affected by chaotropic agents, and the latter is sensitive to molecular oxygen. In the presence of chaotropic agents, Tiron reacts with all of the iron atoms in adrenodoxin to produce its Fe^{3+} complex. The Fe^{3+}–Tiron formed is not reduced by the intramolecular reductants. Accordingly, the reaction is not affected by molecular oxygen. These results are consistent with the implication that all of the iron atoms in adrenodoxin are ferric. In addition to this, the iron atoms in adrenodoxin are less accessible to the chelating agents than those in bacterial ferredoxin.

In general, ferredoxin serves as a redox intermediate of electron-transfer reactions in a variety of physiological systems. A ferredoxin-like protein, adrenodoxin, is a redox intermediate in the NADPH-dependent steroid hydroxylases of adrenal mitochondria. This protein has 2 g-atoms of iron and 2 moles of labile sulfur per mole of protein. The detailed properties have been reviewed previously (Kimura, 1968a).

The reactivity of clostridial ferredoxin with iron-chelating agents has been studied to some extent by Malkin and Rabinowitz (1967). Their work showed that the iron atoms are not readily accessible to chelating agents; however, in the presence of molecular oxygen or chaotropic agents these reagents chelate the iron atoms in ferredoxin.

There are some similarities and dissimilarities among ferredoxins in terms of their iron contents, amino acid sequences, and valence states of iron. Thus, it is of interest to compare the chelator accessibility of adrenodoxin with that of bacterial ferredoxin. Furthermore, three recent physical approaches to the iron valence state on ferredoxins strongly suggest that both iron atoms in adrenodoxin and spinach ferredoxin are ferric (Eaton *et al.*, 1971; Johnson *et al.*, 1971; Poe *et al.*, 1971), whereas 6–8 iron atoms in bacterial ferredoxin are mixtures of Fe^{3+} and Fe^{2+} ions (Blomstrom *et al.*, 1964).

Because in the previous studies by Malkin and Rabinowitz (1967) the authors had difficulty interpreting their results due to lack of information on the valence states of iron atoms in ferredoxin, and also due to the multiplicity of iron content and valence states, it is timely to reinvestigate the reactivity of adrenodoxin with iron chelators. In this paper, some newer interpretations on the reactivities will be presented.

Materials and Methods

Bovine adrenodoxin was prepared as described previously (Kimura, 1968a). The ratio of A_{414} to A_{276} of the preparations used in this investigation was 0.76–0.83, and the iron content was 1.9 g-atoms of iron/mole of protein, assuming a molecular weight of 13,000. The purity of the sample was regarded as more than 90%. An NADPH diaphorase (adrenodoxin reductase) was prepared by the method reported elsewhere (Kimura and Suzuki, 1967).

Urea, guanidine·HCl (Ultra Pure grade), *o*-phenanthro-

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