Influence of Salt, Substrate, and Cofactor Concentrations on the Kinetic and Mechanistic Behavior of Phosphoglycerate Mutase*

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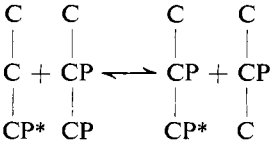
ABSTRACT: Kinetic studies on chicken muscle phosphoglycerate mutase have shown that the reaction mechanism is Ping-Pong both at low ionic strength, where the cofactor diphosphoglycerate may dissociate from the enzyme only once in 100 catalytic cycles, and in the presence of 0.4 M KCl, where the cofactor dissociates much faster than the catalytic rate; in addition, competitive substrate inhibition by both substrates is observed.

The mechanism is thus the same under both sets of conditions; only the rate of dissociation of the coenzyme (together with its dissociation constant) varies greatly with ionic strength. Michaelis constants for 2,3-diphosphoglycerate and 3-phosphoglycerate were 1.4 μM and 0.6 mM at low ionic strength, and 42 μM and 4.6 mM in the presence of 0.4 M KCl; their dissociation constants as dead-end inhibitors were 2.5 and 2.9 mM, and 10 and 6.7 mM under the two sets of conditions. Salt also produces inhibition competitive with 2,3-phosphoglycerate.

The biological role of 2,3-P-glycerate[1] had been open to speculation until it was shown that it behaved as an intermediate in the enzymatic interconversion of 3-P-glycerate and 2-P-glycerate (Sutherland *et al.*, 1949). The stimulation of P-glycerate mutase by 2,3-P-glycerate was interpreted, by analogy with phosphoglucomutase (Leloir *et al.*, 1948), to indicate that 2,3-P-glycerate donated phosphate to 3-P-glycerate according



(1)

to reaction 1. This formulation presumes that [^{32}P]3-P-glycerate is converted into 2,3,-P-glycerate so that the latter compound should become labeled during the reaction. Such a mechanism could either involve the simultaneous presence of both reactants on the enzyme surface (a sequential mechanism), or the presence of only one at a time (a Ping-Pong mechanism). In the latter case, one would expect the enzyme to exist in both phospho and dephospho forms, while no phosphoenzyme is expected in the sequential mechanism. Kinetic studies can distinguish between these possibilities, since a sequential mechanism leads to intersecting reciprocal plots when one substrate is varied at several fixed levels of the other, while a Ping-Pong mechanism gives a parallel line pattern. It is also possible that 2,3-P-glycerate acts as an activator rather than a coenzyme as in equation 1, in which case it would not become labeled during the reaction, and sequential kinetics would be expected.

Previous studies have shown a change in the extent of incorporation of label from [^{32}P]3-P-glycerate into 2,3-P-glycerate with ionic strength for both muscle and yeast mutases (Cascales and Grisolia, 1966). At low ionic strength, ^{32}P is found almost entirely in 2-P-glycerate, and almost no label appears in 2,3-P-glycerate. As the ionic strength is raised, 2,3,-P-glycerate is more extensively labeled, and between 0.2 and 0.4 M KCl its specific activity begins to exceed that of 2-P-glycerate; at 1 M and higher KCl, the incorporation into 2,3-P-glycerate is very high, while the incorporation into 2-P-glycerate becomes negligible.

The maximum rate of exchange into 2,3-P-glycerate induced by salt occurs between pH 7 and 8, and the pH profile does not agree with that of the catalytic reaction where the optimum pH is from 1 to 3 pH units lower (muscle and yeast, respectively), but resembles that of the non-2,3-P-glycerate-dependent mutases. It was therefore of interest to investigate the effect of ionic strength in more detail in order to determine the true mechanism of the reaction. This paper presents a study of both the kinetics and the stability of P-glycerate mutase at a wide range of salt, of 3-P-glycerate, and of 2-3,P-glycerate concentrations.

Experimental Procedure

Reagents and Methods. 3-P-glycerate, free of 2,3-P-glycerate, was prepared by the method of Towne et al.

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[1] Abbreviations used: 2,3-P-glycerate, 2,3-phosphoglycerate; PEP, phosphoenolpyruvate; PGA, phosphoglycerate.

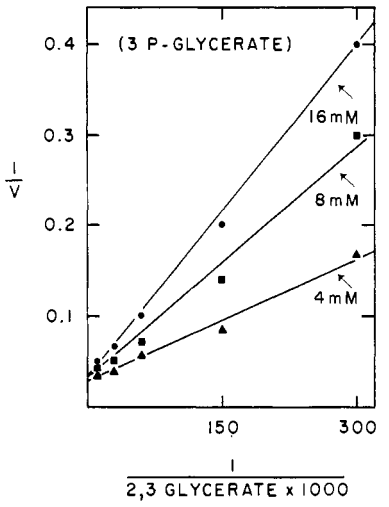


FIGURE 1: Initial velocities at high 3-P-glycerate levels. The conditions were as given in Table I, except that the concentrations of 3-P-glycerate and 2,3-P-glycerate were changed as shown.

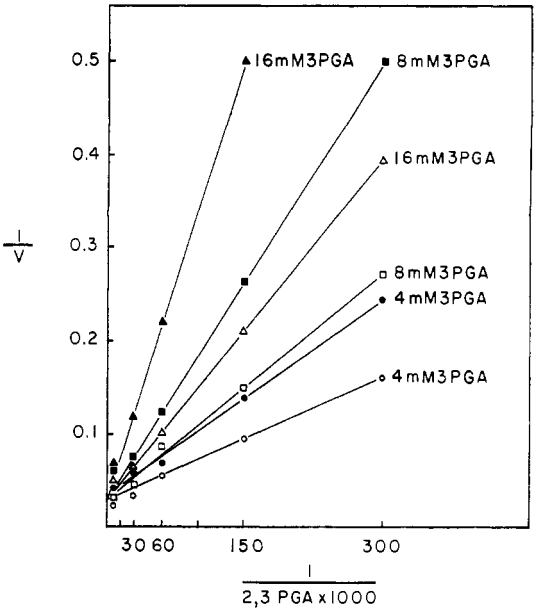


FIGURE 2: The influence of salt on velocity at several levels of 3-P-glycerate. The conditions were as given in Table I except for the concentrations of the reactants which were varied as shown. Open symbols: no added salt; closed symbols: 200 mM KCl added.

(1957). 2,3-P-glycerate prepared according to the method of Grisolia and Joyce (1958) was used. Tris (reagent grade) was obtained from Sigma.

Spectrophotometric measurements were performed in a Gilford automatic multiple-sample recording spectrophotometer, Model 2000, equipped for measurements at constant temperature. 2,3-P-glycerate was assayed according to the method of Jacobs and Grisolia (1966). Protein was determined by a semimicrobiuret method (Rodwell *et al.*, 1957).

Enolase was prepared and assayed as described previously (Grisolia, 1962). Chicken muscle phosphoglycerate mutase was purified by the method of Torralba and Grisolia (1966). Mutase activity was measured with the enolase-coupled assay, which was modified by increasing the amount of enolase to 10 units. One P-glycerate mutase unit is the amount of enzyme that

TABLE I: Influence of Anions and Cations on P-glycerate Mutase at "Low" and at "High" 2,3-P-glycerate Levels.[a]

	% Activity at	
Addn (μmoles)	6.6 μM 2,3-PGA	3.3 mM 2,3-PGA
None	100	137
KCl (600)	62 (77)	164 (170)
KBr (600)	42	143
KNO_3 (60)	75	131
Tris-Cl^- (600)	64	220
Tris-SO_4^{2-} (300)	2	116
$(NH_4)_2SO_4$ (300)	2	116

[a] The conditions were as follows: 0.5 unit of mutase, 10 μmoles of 3-P-glycerate, 10 μmoles of $MgSO_4$, 100 μmoles of Tris-Cl, and 10 units of enolase. Final volume, 3 ml; temperature 30°. When indicated salts were added as recorded and the velocities were measured in the presence of 6.6 μM and 3.3 mM 2,3-P-glycerate. There were linear rates in all cases and the velocities were measured for as long as 15 min. The figures in parentheses indicate data from a second experiment.

TABLE II: Effect of Heat and Salt on Chicken Breast Muscle P-glycerate Mutase Activity.[a]

Additions		°C		
Salt	2,3-P-glycerate	55–57	58–60	62–64
		% Activity		
	−	5.25	0.0	0.0
	+	137.0	132.0	104.0
NaCl	−	47.5	8.5	0.0
NaCl	+		123.0	93.0
KCl	−	73.0	31.0	0.0
KCl	+	144.0	133.0	134.0
$(NH_4)_2SO_4$	−	4.2	0.6	0.0
$(NH_4)_2SO_4$	+	137.0	113.0	58.0
K_2SO_4[b]	−	58.0	9.7	0.0
K_2SO_4[b]	+	122.0	116.0	95.0

[a] The conditions were as follows: 0.05 mg of mutase, 100 μmoles of the indicated salt, and when added 2 μmoles of 2,3-P-glycerate at pH 7.4 in 0.3 ml were heated at the indicated temperatures for 5 min. After cooling all samples were diluted to 1 ml. Mutase activity was measured by the enolase-coupled method. [b] Only 50 μmoles was added.

TABLE III: Velocities with Several Concentrations of 3-P-glycerate and 2,3-P-glycerate at Low Salt Concentration.[a]

2,3-P-glycerate (μM)	3-P-glycerate Added (mM)					
	1	2	5	10	20	40
	Absorbancy Change/min					
0.15	0.009	0.008	0.005	0.004	0.002	0.002
0.5	0.014	0.013	0.007	0.005	0.005	0.002
1.0	0.021	0.020	0.013	0.011	0.007	0.003
5.0	0.038	0.039	0.033	0.027	0.025	0.011
10.0	0.042	0.045	0.041	0.041	0.037	0.022
50.0	0.046	0.051	0.054	0.060	0.069	0.059
100.0	0.046	0.053	0.056	0.068	0.090	0.096
500.0	0.046	0.048	0.063	0.069	0.100	0.126
1000.0	0.038	0.048	0.059	0.069	0.097	0.120
2000.0	0.033	0.043	0.059	0.066	0.090	0.120
4000.0	0.025	0.041	0.053	0.062	0.082	0.120
6000.0	0.022	0.038	0.052	0.062	0.079	0.105

[a] Each cell contained in 3.0 ml 50 μmoles of Tris-Cl^- (pH 7.4), 25 units of enolase, 10 μmoles of $MgCl_2$, the indicated amounts of 3-P-glycerate or 2,3-P-glycerate, and the enzyme (added in 0.1 ml). 50 μl containing the indicated 2,3-P-glycerate at 30° was added to start the reaction. Incubation was at 30°. Under the standard conditions of assay (Grisolia, 1962), the enzyme added gave an absorbancy change of 0.04/min. One unit corresponds to an absorbancy change of 0.1 at 240 mμ in 1 min at 30°. There were slight induction periods as follows: with 40 mM 3-P-glycerate and 5 μM 2,3-P-glycerate there was an induction period of 0.5 min; with 10–6000 μM 2,3-P-glycerate induction was for 1.0 min; with 20 mM 3-P-glycerate, 50–6000 μM 2,3-P-glycerate gave an induction of 0.5 min. All initial velocities were measured from less than 0.2 total absorbancy change. All figures were taken from the initial velocity unless an induction period was present; they are given as changes in absorbancy per minute.

causes an increase of 0.1 absorbance at 240 mμ in 1 min at 30° in a 3-ml cell with a 1-cm light path (Grisolia, 1962). The amount of 2-P-glycerate formed may be calculated from the measured value for PEP. Under our experimental conditions, 1.5 μmoles of 2-P-glycerate formed corresponded to 1 μmole of PEP measured. The molar extinction coefficient of PEP at 240 mμ was taken as 1.75×10^3.

Data Processing. The kinetic nomenclature used in this paper is that of Cleland (1963a). After preliminary graphical analysis indicated that the basic initial velocity pattern was a parallel one (that is, reciprocal plots for one substrate at different levels of the other are parallel), but that both substrates gave competitive substrate inhibition at high concentrations, the data from Tables III and IV (except where an induction period was observed, see below) were fitted to eq 2 by the least-squares method, assuming equal variances for experimental velocities (Wilkinson, 1961). The calculations were performed by a digital computer using a Fortran program (Cleland, 1963b, 1967) which provides values of the kinetic constants and standard errors of these estimates.

$$v = \frac{VAB}{K_aB\left(1 + \frac{B}{K_{Ib}}\right) + K_bA\left(1 + \frac{A}{K_{Ia}}\right) + AB} \quad (2)$$

Results

While the experiments shown here were carried out with chicken breast mutase, similar findings were also obtained with yeast mutase; for brevity, only the findings with the chicken breast mutase are illustrated. Early attempts to explore the kinetic behavior of 3-P-glycerate mutase were impeded by apparent inhibition with increasing concentrations of 3-P-glycerate, as seen in Figure 1. It appeared possible that salt might have been responsible for these effects, since the 3-P-glycerate was

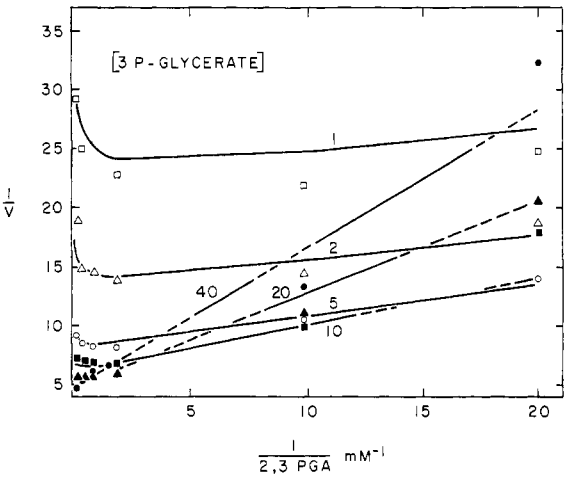


FIGURE 3: The influence of substrate and cofactor concentrations on velocity at high ionic strength. The conditions are as recorded in Table IV. The solid lines represent a least-squares fit of the data to eq 2. 3-P-glycerate: (●), 40 mM, (▲) 20 mM, (■) 10 mM, (○) 5 mM, (△) 2 mM, and (□) 1 mM.

TABLE IV: Velocities with Several Concentrations of 3-P-glycerate and 2,3-P-glycerate in the Presence of 0.4 M Potassium Chloride.[a]

2,3-P-glycerate (μM)	3-P-glycerate Added (mM)					
	1	2	5	10	20	40
	Absorbancy Change/min					
0.15	0.003	0.006	0.002	0.002	0.001	0.001
0.5	0.004	0.007	0.002	0.002	0.001	0.001
1.0	0.007	0.007	0.005	0.003	0.002	0.001
5.0	0.021	0.022	0.015	0.011	0.009	0.004
10.0	0.028	0.029	0.024	0.012	0.016	0.009
50.0	0.040	0.053	0.070	0.055	0.048	0.031
100.0	0.045	0.069	0.094	0.097	0.092	0.075
500.0	0.044	0.072	0.120	0.148	0.164	0.144
1000.0	0.040	0.068	0.118	0.146	0.170	0.164
2000.0	0.040	0.067	0.116	0.139	0.171	0.177
4000.0	0.034	0.053	0.108	0.137	0.176	0.208
6000.0	0.032	0.522	0.098	0.135	0.190	0.216

[a] Each cell contained in 3.0 ml 50 μmoles of Tris-Cl^-, 25 units of enolase, 10 μmoles of $MgCl_2$–0.4 M KCl, the indicated amounts of 3-P-glycerate or 2,3-P-glycerate, and the enzyme. 2,3-P-glycerate was warmed to 30° before adding to the cells to start the reaction. The enzyme used gave an absorbancy change of 0.032/min (0.3 unit under the standard conditions of assay (Grisolia, 1962), but in the presence of 0.4 M KCl) and an absorbancy change of 0.08/min in the standard assay without salt. One unit corresponds to an absorbancy change of 0.1 at 240 mμ in 1 min at 30°. There were induction periods with the following: with 1, 2, 5, and 10 mM 3-P-glycerate, 6 mM 2,3-P-glycerate gave induction periods of 0.5 min with 20 mM 3-P-glycerate, 50–2000 μM 2,3-P-glycerate gave induction periods of 0.5 min; 4000 and 6000 μM 2,3-P-glycerate gave inductions of 1.0 min. With 40 mM 3-P-glycerate, 5–50 μM 2,3-P-glycerate gave inductions of 0.5 min; 100–2000 μM 2,3-P-glycerate gave induction periods of 1.0 min; 4000 and 6000 μM 2,3-P-glycerate gave induction periods of 1.5 min. All figures were taken from the initial velocities unless there was an induction period; they are given as change in absorbancy per minute.

added as the potassium salt and in addition contained added KCl. Indeed, as shown in Figure 2, KCl behaves as an essentially competitive inhibitor for P-glycerate mutase, but 3-P-glycerate still gives competitive substrate inhibition. Other cations and anions were then checked for action on P-glycerate mutase at several levels of 2,3-P-glycerate. At the low levels commonly used to assay the enzyme, salts inhibit mutase action, as shown in Table I. The nature of the anion seems more important than the cation, and sulfate ions are remarkably inhibitory. As expected for competitive inhibition, when the enzyme is saturated with 2,3-P-glycerate, essentially no inhibition by salt is detected; however, under these conditions, in some cases, salt stimulates the reaction. As shown in Table II, salt also remarkably protects the enzyme against heat denaturation. On the other hand, as illustrated previously with the preparation of enolase free of P-glycerate mutase, potassium serves to inactivate residual mutase activity with ethanol (Grisolia *et al.*, 1967).

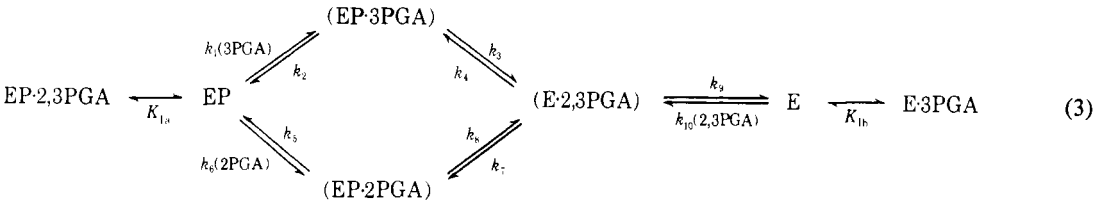
The effect of substrate and cofactor concentration was then further studied both at low ionic strength and in the presence of 0.4 M KCl. In view of the wide concentration ranges, the data are presented in tabular form in Tables III and IV. A typical plot of a portion of the data obtained in the presence of 0.4 M KCl is shown in Figure 3, and serves to illustrate the kinetic patterns observed. The competitive substrate inhibition by 3-P-glycerate seen in Figures 1 and 2 is also prominent here (the slopes and not the intercepts of the plots are affected by high 3-P-glycerate). In addition, 2,3-P-glycerate causes substrate inhibition which also appears competitive (the upward curvature of the plots near the left vertical axis is more prominent at low 3-P-glycerate levels and disappears at high 3-P-glycerate). These substrate inhibitions obscure the basic kinetic pattern somewhat, but it is clear from the data in the region where both substrate concentrations are low that the basic pattern is a series of parallel lines, *i.e.*, that characteristic of a Ping-Pong mechanism. The same pattern was observed both with high and low salt, although the observed values of the kinetic constants were markedly altered by salt.

Since the kinetic pattern in both cases appeared to be a series of parallel lines upon which was superimposed competitive substrate inhibition by both substrates, the data were fitted to eq 2, which corresponds to this pattern. Good fits were obtained in both cases, and the resulting constants and the standard errors of their estimates are given in Table V.

Discussion

The present findings, taken in conjunction with pre-

vious investigations, indicate that both the kinetic behavior and the structural stability of P-glycerate mutase are affected by cofactor and salt. At both low and high ionic strength the initial velocity patterns are the same and show a basic Ping-Pong pattern with competitive substrate inhibition by both substrates (eq 2), although the numerical values of some of the kinetic constants are greatly altered by salt. However, the isotopic evidence shows that at low ionic strength, ^{32}P from 3-P-glycerate is found almost entirely in 2-P-glycerate, while at high ionic strength it is nearly all found in 2,3-P-glycerate (Cascales and Grisolia, 1966). These data are all consistent with the mechanism shown below, and rule out possible change of Ping-Pong to sequential mechanisms, such as were considered earlier when the concentration of 2,3-P-glycerate was changed (Grisolia and Uberlaker, 1967). In eq 3 E and EP represent free



(3)

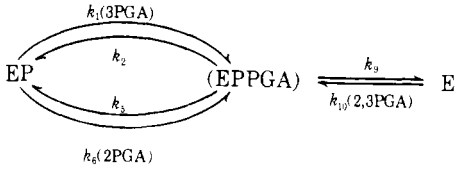
and phosphoenzyme; EP·2,3-PGA and E·3-PGA are dead-end complexes of EP with 2,3-P-glycerate and of E with 3-P-glycerate; and (EP·3-PGA), (EP·2-PGA), and (E·2,3-PGA) are mutually interconvertible central complexes[2] which may dissociate to 3-P-glycerate plus EP, 2-P-glycerate plus EP, and 2,3-P-glycerate plus E, respectively. This mechanism predicts the observed initial velocity patterns which correspond to eq 2.

The isotope studies can be explained if one assumes that at low ionic strength k_9 is small compared to some of the unimolecular constants determining the maximum velocity (specifically, that k_9 is much less than $k_5k_7/(k_5 + k_8)$, since the ratio of isotope transfer from 3-P-glycerate to 2,3-P-glycerate to over-all chemical reaction to form 2-P-glycerate is given by $k_9(k_5 + k_8)/(k_5k_7)$), but that at high ionic strength k_9 becomes much larger than the maximum velocity. Presumably k_{10} increases also, but to a lesser extent, since K_a rises by a factor of only 30-fold (Table V). Thus, at low ionic strength 2,3-P-glycerate may dissociate from the enzyme only once in every 100 catalytic cycles (based on the isotopic data of Cascales and Grisolia, 1966), although its presence in the system is still needed to prevent gradual pile up of free E, and possibly to convert the E initially present into EP, which is the form that reacts with the monophosphate. At high ionic strength, the dissociation of 2,3-P-glycerate is so much faster than the maximum velocity that the (E·2,3-PGA) complex exchanges completely with free 2,3-P-glycerate, and no label finds its way directly into 2-P-glycerate.

This mechanism is basically the same as that proposed by Ray for phosphoglucomutase (Ray and Roscelli, 1964). Under the conditions he used, glucose 1,6-diphosphate dissociated about once in 20 catalytic cycles, but no attempt was made to study the effect of ionic strength on this rate. A mechanism of this type, where substrate and coenzyme display apparent Ping-Pong kinetics, although the coenzyme need not dissociate as an obligatory step in every catalytic cycle, may well be a universal one for coenzyme-mediated mutases. However, since no stable phospho form has been isolated to date, the phosphate group of phosphoglyceromutase may not be covalently bound to the hydroxyl group of a serine residue as it is the case in phosphoglucomutase.

The kinetic constants in Table V allow comparison of the binding of 3-P-glycerate or 2,3-P-glycerate to EP subject to the following restrictions. K_{Ia} and K_{Ib} are dissociation constants for dead-end complexes and are thus true equilibrium constants; K_a is the dissociation constant of 2,3-P-glycerate from the grouped central complexes under conditions where no EP exists (3-P-glycerate saturating), and K_b is the dissociation constant of 3-P-glycerate from the grouped central complexes where no free E exists (2,3-P-glycerate saturating),[3] *i.e.*, as with all Michaelis constants, K_a and K_b are dynamic dissociation constants of 2,3-P-glycerate and 3-P-glycerate from the central complexes in the steady state under stated conditions rather than true equilibrium constants. If these two types of constants can be compared, it appears that binding of the basic 3-P-glycerate group itself, as in the dead-end complexes, provides a dissociation constant around 2 or 3 mM at low ionic strength. (The 2-phosphate of the coenzyme

[2] This mechanism can be written with arrows connecting (EP·3-PGA) and (EP·2-PGA) directly without altering the form of the rate equation, or any of the conclusions of this paper. It is also possible to write the center portion of the mechanism (omitting the dead-end complexes for simplicity) as



where (EPPGA) represents all enzyme in the form of central complexes. On the other hand, the number of central complexes in the mechanism can be expanded with no limit. The formulation in the mechanism was chosen because it emphasizes the various forms that the bonds in the central complexes may take, and the molecules which will result from the dissociation of such complexes.

[3] That is, it is defined by the relationship

$$K_b = \frac{[B][\text{concentration of EP}]}{[\text{concentration of central complexes}]}$$

which holds under all steady-state conditions.

TABLE V: Kinetic Constants for 3-P-glycerate and 2,3-P-glycerate with Chicken Breast Phosphoglycerate Mutase.[a]

Substrate	Kinetic Constant	Low Ionic Strength (μM)[b]	At 0.4 M KCl (μM)[c]
2,3-PGA	K_a	1.4 ± 0.3	42 ± 8
	K_{Ia}	2,500 ± 400	10,000 ± 2,000
3-PGA	K_b	600 ± 60	4,600 ± 300
	K_{Ib}	2,900 ± 800	6,700 ± 1,700

[a] The calculated maximum velocities were 8000 ± 300 units/mg of enzyme for the experiments recorded in Table III (low ionic strength) and 6600 ± 300 units/mg of enzyme for the experiments recorded in Table IV (at 0.4 M KCl). These values are 3.33 and 2.65 times higher than those obtained under the standard conditions of assay (Grisolia, 1962). Also, they may be converted into micromoles of 3-P-glycerate turned over by dividing the units by 3.9 (Jacobs and Grisolia, 1966). Since the molecular weight of chicken P-glycerate mutase is 65,690 (Torralba and Grisolia, 1966), the absolute values will be 146,000 (low ionic strength) and 112,000 moles (at 0.4 M KCl) of 3-P-glycerate turned over/mole of enzyme per min. [b] The ionic strength was ~0.03 to ~0.15 from the lowest to the highest substrate and cofactor additions. [c] The ionic strength was ~0.43 to ~0.55 from the lowest to the highest substrate and cofactor additions.

should be excluded by the phosphate of EP in the dead-end complex, EP·2,3-PGA, and should not contribute to binding in this complex). The presence of a phosphate group in a reactive position on EP allows tighter binding of 3-P-glycerate by a factor of 4 or 5; when both phosphates of 2,3-P-glycerate contribute to binding to free E, the binding is enhanced some 2000-fold.

At high ionic strength, all binding is looser, and the binding of 2,3-P-glycerate to E, which involves two phosphates instead of only one as in the above three cases, is affected the most. High ionic strength would be expected to raise the unimolecular rate constant for dissociation and thus the values of the dissociation constants. Since the maximum velocity is affected very little by ionic strength, it appears likely that k_5 in the mechanism outlined above is not the rate-limiting step, but rather that some bond-breaking step is the rate-limiting one. It is also clear from the isotopic data that k_9 is increased (perhaps by 10^4-fold) much more by high ionic strength than the factor of 30 by which K_a is increased. Perhaps high ionic strength has altered the conformation of the enzyme somewhat so that both the association and dissociation of 2,3-P-glycerate are enhanced.

It should be noted that the physiological concentration of 2,3-P-glycerate in the red cell is probably misleading insofar as extrapolation to the physiological concentration in other tissues. The concentration in the red cell of those species which possess 2,3-P-glycerate is roughly 2–8 mM, and is in all likelihood related to conformational effects on hemoglobin as previously suggested, a suggestion which has been reinforced by recent findings of ourselves and others (Grisolia, 1968; Benesch and Benesch, 1967; Chanutin and Curnish, 1967). As shown in this paper, the K_m's for 2,3- and 3-P-glycerate are in the micromolar and millimolar range, respectively, even at salt concentrations above those considered physiological, and in agreement with the meager data available in the literature for 3-P-glycerate (Hill and Mills, 1961; Arese *et al.*, 1967). It should also be noted that our own measurements revealed that the concentration of 2,3-P-glycerate in many tissues is also in the millimolar range but lower than in the red cell, *whether or not* the species has 2,3-P-glycerate in its erythrocytes (Grisolia and Joyce, 1959). It is also of interest that the diphosphoglycerate mutase appears to be present in fairly large quantities only in muscle, brain, and in the erythrocyte. Further, the quantity of this enzyme in muscle or in the erythrocyte has no relation to the concentration of 2,3-P-glycerate in the erythrocyte. For example, both chicken and pigeon tissues contain a very large amount of diphosphoglycerate mutase, and yet, they lack diphosphoglycerate in their red cells.

Acknowledgments

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Studies on Acid Deoxyribonuclease. V. The Oligonucleotides Obtained from Deoxyribonucleic Acid and Their 3′-Phosphate Termini*

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ABSTRACT: Deoxyribonucleic acid (DNA) samples from different sources have been digested with spleen acid deoxyribonuclease. An initial fast phase followed by a terminal very slow one can be recognized in both the hyperchromic shift and the acid-soluble oligonucleotide release taking place during the digestion. DNase digests in the early slow phase are composed of oligonucleotides having an average size of 10–12. Over 65% of the digest is made up of fragments of a size larger than 7, which cannot be resolved by DEAE-cellulose–urea columns. The 3′-phosphate-terminal nucleotides have been determined after digestion with spleen acid phosphomonoesterases I and II and spleen exonuclease, using several different techniques. Deoxyguanylic and deoxyadenylic acids, in about equal amounts, form about 80% of the 3′-phosphate termini; thymidilic and deoxycytidylic acids form 10 to 15 and 5 to 7% of the 3′-phosphate ends, respectively. Various kinds of artifacts explaining the widely different results reported by previous authors have been investigated.

Spleen acid deoxyribonuclease (deoxyribonucleate 3′-oligonucleotidohydrolase (EC 3.1.4.6): deoxyribonuclease II) has been the object of extensive investigations in this laboratory over the past few years. These have shown that the enzyme is able to split simultaneously both DNA strands at the same level (Bernardi and Sadron, 1961, 1964a,b; Mac Hattie *et al.*, 1963) and is competitively inhibited by tRNA, rRNA, and certain synthetic polyribonucleotides (Bernardi, 1964; Jacquemin-Sablon *et al.*, 1964). Spleen acid DNase has been isolated as a homogeneous protein (Bernardi *et al.*, 1963, 1966; Bernardi and Griffé, 1964), characterized in its physical and chemical properties (Bernardi *et al.*, 1965), and shown to be a dimeric protein molecule (Bernardi, 1965). The enzyme can split very slowly bis(*p*-nitrophenyl)phosphate and the *p*-nitrophenyl esters of deoxyribonucleoside 3′-phosphates (Bernardi and Griffé, 1964), but not those of the 5′ derivatives (Bernardi, 1966); in spite of this "phosphodiesterase" activity, the enzyme has no exonuclease activity as first shown by Bernardi and Griffé (1964) and later confirmed by the present work. Acid DNase is a very widely distributed lysosomal enzyme, probably present in all the cells of eukariotic organisms (Cordonnier and Bernardi, 1965; C. Cordonnier and G. Bernardi, to be published).

The specificity of spleen acid deoxyribonuclease has been investigated in several laboratories (Privat de Garilhe and Laskowski, 1954; Laurila and Laskowski, 1957; Koerner and Sinsheimer, 1957; Vanecko and Laskowski, 1961, 1962; Doskocil and Sorm, 1961a,b, 1962). However, in spite of the remarkable efforts devoted to solve this problem, no clear picture of the basic properties of the digest, such as average size and terminal nucleotides, has emerged so far.

In view of this, we decided to investigate this problem thinking that we were in a better position than previous authors for the following reasons: (a) the availability of homogeneous spleen acid DNase preparations (free of exonuclease activity) having a specific activity about three times higher than the best preparations previously used by other workers (Bernardi and Griffé, 1964; Bernardi *et al.*, 1965, 1966); (b) the availability of three

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