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REVERSE AND FORWARD REACTIONS OF CARBAMYL PHOSPHOKINASE FROM *STREPTOCOCCUS FAECALIS* R

PARTICIPATION OF NUCLEOTIDES AND REACTION MECHANISMS

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The participation of Mg complex of nucleoside diphosphates and nucleoside triphosphates in the reverse and forward reactions catalyzed by purified carbamyl phosphokinase (ATP carbamate phosphotransferase, EC 2 7 2 2) of *Streptococcus faecalis* R, ATCC-8043 were studied The results of initial velocity studies at approx 1 mM free Mg^{2+} concentration have indicated that in the reverse reaction MgdADP was as effective a substrate as MgADP The phosphoryl group transfer from carbamyl phosphate to MgGDP, MgCDP and MgUDP was also observed at relatively higher concentrations of the enzyme and respective magnesium nucleoside diphosphate In the forward direction MgdATP was found to be as efficient a phosphate donor as MgATP On the other hand, Mg complexes of GTP, CTP and UTP were ineffective even at higher concentrations of the enzyme and respective magnesium nucleoside triphosphate Product inhibition studies carried out at non-inhibitory level of approx 1 mM free Mg^{2+} concentration have revealed that the enzyme has two distinct sites, one for nucleoside diphosphate or nucleoside triphosphate and the other for carbamyl phosphate or carbamate, and its reaction with the substrates is of the random type Further tests of numerical values for kinetic constants have indicated that they are partially consistent with the Haldane relationship which is characteristic of rapid equilibrium and random mechanism

Introduction

The enzymatic formation of carbamyl phosphate (carbamyl-*P*) has been shown [1,2] to be brought about by two different reactions

$$\text{I}\quad NH_4^+ + HCO_3^- + 2ATP \xrightarrow[\substack{N\text{-acetylglutamate}\\ \text{or L-glutamine}}]{Mg^{2+}} \text{Carbamyl-}P + 2ADP + P_i$$

$$\text{II}\quad NH_2COO^- + ATP \rightleftharpoons ADP + \text{carbamyl-}P$$

The reaction I catalyzed by carbamyl phosphate synthetase is essentially irreversible favouring formation of carbamyl-*P* [3] It is activated by *N*-acetylglutamate or L-glutamine The enzyme is present in vertebrates, various bacteria and fungi [4–9] The reaction II is catalyzed by carbamyl phosphokinase This enzyme was discovered first in *S faecalis* [10] and later in several bacterial species [1,11,12] The equilibrium of reaction II is in the reverse direction favouring the formation of ATP [13] It has been reported that for the reverse reaction of this enzyme, ADP cannot be replaced by other nucleoside diphosphates, and for the forward reaction, ATP cannot be replaced by other nucleoside triphosphates [14] We have, however, found that MgdADP can serve as an effective substrate for the reverse reaction of the enzyme and MgdATP for the forward reaction

Further at relatively high concentrations, the Mg complex of GDP, CDP and UDP was also found to be utilized by carbamyl phosphokinase in place of MgADP in the reverse reaction These studies form the subject matter of the present paper The paper also includes results of the studies on kinetic behaviour and the reaction mechanism of this enzyme These studies were carrried out by using each of the reactants of the forward reaction as a product inhibitor of the reverse reaction, and vice versa, under the conditions in which free Mg^{2+} was maintained at 1 mM level The results indicate that the reaction of carbamyl phosphokinase with the substrates exhibits rapid equilibrium and is of random type

Materials and Methods

Sodium salts of nucleoside diphosphates and nucleoside triphosphates were obtained from Sigma Chemical Company, U S A The nucleotides were checked for their purity by paper chromatography in isobutyric acid/NH_3/water (66 1 33, v/v) [15] Those batches which showed a single spot on the chromatogram under ultraviolet light were used directly without any further purification while those which were found to contain impurities were purified by preparative ascending paper chromatography on Whatman No 3 paper using the same solvent system The relevant band of the nucleotide on the chromatogram was detected by ultraviolet light, cut and eluted with water and then lyophilized The lyophilized preparations were rehydrated and the concentrations were determined by measurement at 259 nm in a Perkin-Elmer 124 Double-Beam Spectrophotometer The stock solutions were adjusted to pH 7 6 with 0 5 M NaOH and kept frozen at −20°C Lithium salt of carbamyl phosphate was purchased from Koch-Light Company [^{32}P]Carbamyl phosphate was prepared according to Jones et al [16] with slight modification as described by Tanaka et al [17] $NaH^{14}CO_3$ (50 mCi/mmol), was obtained from the Isotope Division of this Research Centre, which was diluted to 0 05 mCi/mmol by $KHCO_3$ before use

Carbamyl phosphokinase (600 units/mg protein) was purified to homogeneity from arginine adapted cells of *S faecalis* according to the procedure described by Marshall and Cohen [18] Ornithine transcarbamylase was isolated from DEAE-cellulose fractions during the purification of carbamyl phosphokinase [18] In the present paper, 1 unit carbamyl phosphokinase or ornithine transcarbamylase is defined as the amount of enzyme capable of forming 1 μmol reaction product/min under the condition of forward reaction [18]

γ-[^{32}P]dATP and γ-[^{32}P]ATP were synthesized enzymatically by carbamyl phosphokinase as described below -Carrier-free [^{32}P]-orthophosphoric acid (2 mCi) in HCl was dried at 75°C and then rehydrated by 100 μl 0 25 M K_2HPO_4 To the above were added 50 μl 1 M sodium acetate buffer, pH 4 9, and 25 μl freshly prepared 2 5 M KCNO After incubation of this mixture for 15 min at 30°C, 25 μl more of 2 5 M KCNO were added and the reactants were further incubated for another 15-min period, and chilled The above procedure of adding KCNO in two installments was resorted to for minimising the decomposition of KCNO and carbamyl phosphate (generated in situ from KCNO and K_2HPO_4 [3,19]) during further incubation under the experimental conditions To the chilled mixture were added 100 μl 1 M Tris-HCl, pH 7 5/200 μl 0 1 M dADP or ADP/ 100 μl 0 17 M $MgCl_2$/20 μg purified carbamyl phosphokinase from *S faecalis* The final volume was adjusted to 2 ml with water The complete reaction mixture was brought to 35°C and incubated at this temperature for 30–40 min The reaction was stopped by placing the tube in an ice bath and γ-[^{32}P]dATP or ATP formed in the reverse reaction catalyzed by carbamyl phosphokinase was isolated by preparative paper chromatography, as described before, using isobutyric acid/ammonia/water (66 1 33, v/v) as the solvent system To determine radioactivity in γ-[^{32}P]dATP and ATP an aliquot was spotted on a Whatman No 1 paper strip, dried by hot-air blower and placed in a glass vial containing 10 ml scintillator fluid (0 5%, 2,5-diphenyloxazole (PPO) in toluene) and the radioactivity counted in a Beckman LS-100 liquid scintillation system

The reverse reaction catalyzed by carbamyl phosphokinase was studied by following the transfer of [^{32}P]phosphate group from [^{32}P]carbamyl phosphate to Mg complex of nucleoside diphosphates In all experiments carbamyl phosphokinase was diluted with 0 05% bovine serum albumin [18] before use The reaction mixture contained in a total volume of

1 ml, Tris-HCl buffer (pH 8 0) 100 mM, varying concentrations of nucleoside diphosphates and [^{32}P]carbamyl phosphate as indicated in the figures, sufficient $MgCl_2$ to give the required concentration of Mg nucleotide complex while maintaining the free Mg^{2+} at approx 1 mM, carbamyl phosphokinase equivalent to 1 μg protein (except for experiments with MgCDP, MgUDP and MgGDP as substrates where 15 μg enzyme protein was used) Incubations were carried out at 30°C for 1–2 min for experiments with MgADP and MgdADP and for 10–15 min with MgCDP, MgUDP and MgGDP The reaction was started by the addition of [^{32}P]carbamyl phosphate and was stopped by the addition of 100 μl 2 5 M $HClO_4$ The tubes were placed in ice and 1 ml 10% aqueous suspension of activated charcoal (Norit-SG, Sigma Chemical Co, U S A) containing 0 2% Triton X-100 was added to adsorb the nucleoside triphosphate formed The suspension was centrifuged and 0 5 ml of the supernatant was counted for radioactivity, as described before, to determine the amount of unreacted [^{32}P]carbamyl phosphate The amount of nucleoside triphosphate formed in the reaction was determined from the specific radioactivity of [^{32}P]carbamyl phosphate Control tubes containing all components (except the enzyme) were also incubated and treated in a similar manner and the enzyme was added to reagent blanks after the addition of the stopping reagent

The contamination of nucleoside diphosphates with ADP and of carbamyl phosphokinase with nucleoside diphosphokinase were tested as follows (1) To test the first possibility, the reaction mixture containing higher concentrations of carbamyl phosphokinase and Mg-nucleoside diphosphate was incubated as described before After stopping the reaction by chilling, 10 μl reaction mixture were chromatographed along with the corresponding nucleoside triphosphate as the standard marker on Whatman paper No 3 using isobutyric acid/ammonia/water The relevant spot was marked under ultraviolet light and was cut and counted for radioactivity as described in the text (2) The second possibility was examined by replacing [^{32}P]carbamyl phosphate by γ-[^{32}P]ATP as the phosphate donor under the similar conditions of reverse reaction At the end of the reaction, 10 μl reaction mixture were chromatographed along with the standard marker as above, and the radioactivity in the relevant spot was measured to detect the phosphoryl group transfer from γ-[^{32}P]ATP to the Mg complex of nucleoside diphosphates

The reaction in the forward direction catalyzed by carbamyl phosphokinase was studied by following the incorporation of the radioactivity from $KH^{14}CO_3$ to citrulline, in the presence of ornithine and ornithine transcarbamylase [20] Each reaction tube contained 50 mM Tris-HCl, pH 8 0, varying concentrations of [^{14}C]carbamate (as prepared by equilibrating a solution containing 0 2 M $KH^{14}CO_3$/0 4 M NH_4Cl [18]) and nucleoside triphosphate as indicated in the figures, sufficient $MgCl_2$ to give the required concentration of Mg-nucleoside triphosphate complex while maintaining the concentration of free Mg^{2+} at approx 1 mM, carbamyl phosphokinase equivalent to 2 μg protein and 100 units ornithine transcarbamylase Incubations were done at 30°C for 2–4 min and the reaction was stopped by the addition of 0 4 ml 2 M HCl in 25% ethanol 200 μl of the acid-treated reaction mixture (containing acid-stable [^{14}C]citrulline formed in the reaction) were spotted on a Whatman paper No 3 strip, dried using a hot-air blower and counted for radioactivity as described earlier

The calculations of Mg concentrations sufficient to convert the nucleotide to its Mg complex, while maintaining the total free Mg^{2+} at 1 mM level, were carried out essentially as described by Morrison and Cleland [21] using the following relationship

$$[M] = \frac{[\mu][S]}{[K] + [\mu]}$$

where [M] = the total concentration of $MgCl_2$ required, [μ] = constant concentration of free Mg^{2+} to be maintained, [S] = total concentration of nucleotide and [K] = dissociation constant for Mg-nucleotide complex The values of the dissociation constants used for MgADP and MgATP were 0 25 mM and 14 7 μM, respectively [22] The calculations of Mg concentration for other nucleoside diphosphates and nucleoside triphosphates were made on the assumption that their Mg complex has the same dissociation constant as that for MgADP and MgATP, respectively [23]

Product inhibition studies in the reverse reaction were carried out by following the transfer of [^{32}P]-phosphate group from [^{32}P]carbamyl phosphate to

MgdADP The unreacted [^{32}P]carbamyl phosphate and the amount of γ-[^{32}P]dATP formed were determined as described before Product inhibition studies in the forward direction were carried out as follows (1) Inhibition in the presence of carbamyl phosphate was studied by following the transfer of [^{32}P]phosphate group from γ-[^{32}P]dATP to carbamate and the amounts of [^{32}P]carbamyl phosphate formed in the reaction were determined after adsorbing the unreacted γ-[^{32}P]dATP on Norit as described before (2) Inhibition of the reaction in the absence of carbamyl phosphate was studied by following the transfer of ^{14}C radioactivity from $KH^{14}CO_3$ to acid-stable citrulline in the presence of ornithine and ornithine transcarbamylase, as described for the studies of forward reactions

Results and Discussion

Participation of Mg-nucleoside diphosphate in the reverse reaction

The studies on initial velocity of the reverse reaction, with each Mg-nucleoside diphosphate as a function of both carbamyl phosphate and Mg-nucleoside diphosphate concentration, have indicated that phosphoryl group transfer occurs when MgADP was replaced with Mg complex of dADP, GDP, CDP and UDP When reciprocal velocities were plotted against reciprocal substrate concentrations, a series of straight lines was obtained which intersect at the left of the vertical axis The crossover points of these lines lay above the horizontal axis for MgADP and MgdADP while they were below the axis for MgGDP, MgCDP and MgUDP Figs 1 and 2 depicting initial velocity data for MgdADP and MgGDP, respectively, could serve as examples for this series of experiments This indicates that the presence of carbamyl phosphate enhances the binding of MgADP or MgdADP to the enzyme and vice versa, while carbamyl phosphate and Mg complexes of GDP, CDP or UDP hinder the binding of one another It follows then that the reaction has a sequential mechanism in which both the substrates react with the enzyme before either product dissociates, and that the mechanism may be ordered or random type since the double-reciprocal plots with different substrate concentrations display the same form of initial velocity pattern Replots of the slopes and vertical intercepts of the double-reciprocal plots against the reciprocal concentration of each Mg-nucleoside diphosphate and carbamyl phosphate were found to be linear Quantitative analysis of data was made and the kinetic constants for each Mg-nucleoside diphosphate and carbamyl phosphate are recroded in Table I The kinetic constants for MgADP and MgdADP are more or less identical but differ widely from those of the Mg

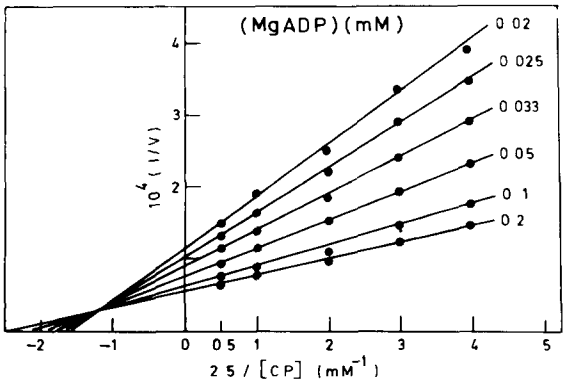

Fig 1 Effect of MgADP concentration on the initial velocity of reverse reaction with carbamyl phosphate (CP) as the variable substrate The concentrations of MgADP are indicated on the lines of the double-reciprocal plot All the points given in the figure are the average of three sets of experiments

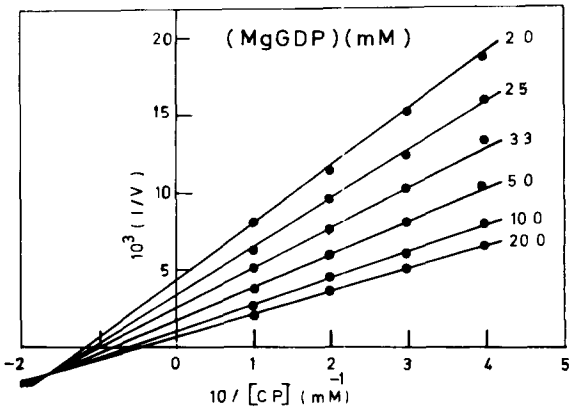

Fig 2 Effect of the concentrations of MgGDP on the initial velocity of reverse reaction with carbamyl phosphate (CP) as the variable substrate The concentrations of MgGDP are indicated on the lines of the double-reciprocal plot All the points given in the figure are the averages of three sets of experiments

TABLE I

KINETIC CONSTANTS AND V FOR THE REACTION OF Mg-NUCLEOSIDE DIPHOSPHATES AND CARBAMYL PHOSPHATE WITH CARBAMYL PHOSPHOKINASE IN THE REVERSE REACTION

The kinetic constants were calculated from the initial velocity data The values given are the averages ± S E of three independent sets of experiments The standard errors of the mean for the results in this and in subsequent tables were calculated as described by Weatherburn [24] NTP, nucleoside triphosphate

Substrate	Michaelis constant K_m (mM)	Dissociation constant K_s (mM)	V(μmol NTP/ μg enzyme per min at 30°C)
MgADP	0 04 ± 0 006	0 06 ± 0 008	0 42 ± 0 014
MgdADP	0 06 ± 0 008	0 14 ± 0 009	0 24 ± 0 016
MgGDP	11 0 ± 1 69	2 5 ± 0 21	0 0026 ± 0 0001
MgCDP	14 0 ± 0 94	2 6 ± 0 18	0 024 ± 0 002
MgUDP	13 3 ± 1 53	4 3 ± 1 18	0 015 ± 0 001
Carbamyl phosphate in the presence of			
MgADP	1 4 ± 0 16	2 0 ± 0 21	
MgdADP	2 0 ± 0 4	4 7 ± 1 07	
MgGDP	16 6 ± 2 11	3 4 ± 0 62	
MgCDP	14 3 ± 1 1	2 0 ± 0 12	
MgUDP	12 5 ± 1 43	4 0 ± 0 49	

complexes of GDP, CDP and UDP The values of Michaelis constants for Mg-nucleoside diphosphates show a large variation from 0 04 mM for MgADP to 14 mM for MgCDP, while dissociation constants vary from 0 06 mM for MgADP to 4 3 mM for MgUDP The values of dissociation constants for carbamyl phosphate in the presence of each Mg-nucleoside diphosphate are relatively constant varying from 2 to 4 7 mM with intermediate values of 3 4 and 4 0 mM, while the values of Michaelis constants show a large variation from 1 4 mM with MgADP to 16 6 mM with MgGDP The maximum velocity with each Mg-nucleoside diphosphate, as recorded in Table I, shows a different pattern to that of the kinetic constants The V values with MgADP and MgdADP are much higher than those with MgGDP, MgCDP and MgUDP

It was found that carbamyl phosphokinase activity with MgdADP and MgADP could be detected at less that 1 μg enzyme concentration while with other Mg-nucleoside diphosphates, a 15-fold higher concentration of the enzyme (15 μg) was essential Further, it was ascertained that the carbamyl phosphokinase preparation was free from nucleoside diphosphokinase contamination since phosphoryl group transfer was not observed when [^{32}P]carbamyl phosphate was replaced by γ-[^{32}P]ATP under the conditions of reverse reaction It was ascertained by paper chromatography that the reactions observed with relatively high concentrations of Mg-nucleoside diphosphates were due to nucleoside diphosphates themselves and not due to contamination of these by ADP

It is pertinent to mention at this point that creatine kinase has also been reported to behave similarly, a 30-fold increase in the creatine kinase concentration is found to be necessary to bring about the reaction with relatively higher concentrations of MgGDP, MgCDP and MgUDP in place of MgADP or MgdADP [23]

The large variation in the velocity of reaction at fixed concentration of Mg-nucleoside diphosphates and carbamyl phosphate and a relatively low concentration of enzyme can thus be ascribed to changes in both the maximum velocity and the affinity of the enzyme for the nucleotide It has been reported that ADP and ATP in the reverse and forward reactions, respectively, cannot be replaced by any of the other nucleoside diphosphates and nucleoside triphosphates (unpublished observation reported in Ref 14) This inference, which is in conflict with the present results, might possibly have been drawn from the experiments carried out with lower concentrations of the enzyme, Mg-nucleoside diphosphate and carbamyl phosphate

Participation of Mg-nucleoside triphosphate in the forward reaction

The studies of initial velocity patterns in the forward direction indicated that amongst the Mg-nucleoside triphosphates tested, only MgdATP could serve as the substrate as efficiently as MgATP The double-reciprocal plots of initial velocity data with MgATP and MgdATP, as a function of both carbamate and nucleotide concentrations, gave a series of straight lines intersecting at the left of the vertical axis with the crossover points lying above the horizontal axis Enzymatic activity could not be detected with MgGTP, MgCTP and MgUTP even at higher concentrations of enzyme (20 μg) and nucleotide The kinetic constants calculated from the initial velocity data are recorded in Table II The affinity of

TABLE II

KINETIC CONSTANTS AND V FOR REACTIONS OF Mg-NIECLEOSIDE TRIPHOSPHATE AND CARBAMATE WITH THE CARBAMYL PHOSPHOKINASE IN THE FORWARD REACTION

The kinetic constants were calculated from the initial velocity data The values given are the averages ± S E of three independent sets of experiments CP, carbamyl phosphate, CPK, carbamyl phosphokinase

Substrate	K_m (mM)	K_s (mM)	V(μmol CP/ μg CPK per min at 30°C)
MgATP	0 62 ± 0 09	1 25 ± 0 14	0 084 ± 0 002
MgdATP	0 5 ± 0 05	1 6 ± 0 17	0 067 ± 0 004
Carbamate (with MgATP)	4 74 ± 0 20	12 5 ± 1 31	
Carbamate (with MgdATP)	3 8 ± 0 12	11 53 ± 0 67	

carbamyl phosphokinase for MgATP and MgdATP is more or less similar, as can be inferred by the dissociation and Michaelis constants in respect of these substrates, while the V value for MgATP is somewhat higher than that for MgdATP

Kalman and Duffield [25] and Marshall and Cohen [18] have reported values for various kinetic constants which are different from the ones reported in the present study (Tables I and II) It may be mentioned here that the conditions under which the constants have been determined in the present study differ from those in the previous studies with respect to pH, temperature, ionic strength and composition of the buffer Further, their kinetic experiments were carried out under the condition where the total Mg^{2+} concentration was far in excess of nucleotide concentration, while in the present experiments the free Mg^{2+} concentration was held constant at approx 1 mM level, it has been shown that higher concentrations of Mg^{2+} are inhibitory to both forward and reverse reactions [18]

Product inhibition and the reaction mechanism

Product inhibition studies at the noninhibitory level of 1 mM free Mg^{2+} were carried out to understand the reaction mechanism of this enzyme. The double-reciprocal plots of inhibition data indicate that MgdATP and MgdADP are competitive inhibitors of each other and this is also true with respect to carbamate and carbamyl phosphate Only the results of competitive inhibition of forward reaction by MgdADP with MgdATP as the variable substrate are illustrated in Fig 3, as an example of such inhibition pattern Replots of the slopes of double-reciprocal plots against the concentrations of respective products as competitive inhibitors were found to be linear The constants obtained from these data have been recorded in Table III Analyses of data with carbamate and MgdADP as product inhibitors indicated that these are non-competitive inhibitors of each other in the reverse and forward reactions, respectively Similar inhibition patterns were also obtained in respect of carbamyl phosphate vs MgdATP in the forward and reverse reactions, respectively An example of such inhibition patterns can be seen in Fig 4, in which the results of the non-competitive inhibition of the reverse reaction by carbamate with MgdADP as the variable substrate are depicted This would imply that in the forward reaction, the central complex enzyme-MgdATP-carbamate is generated to be transformed into enzyme-MgdADP-carbamyl phosphate complex In the reverse reaction the central complex enzyme-MgdADP-carbamyl phosphate is generated and transformed into enzyme-MgdATP-carbamate complex In both the forward and reverse reactions two 'dead-end' products, viz , enzyme-MgdADP-carbamate and enzyme-MgdATP-carbamyl phosphate, are also formed Replots of the slopes of double-reciprocal plots of inhibition data against the concentrations of respective products as non-competitive inhibitor are linear, the constants obtained from these data are recorded in Table IV

The observed patterns of product inhibition would suggest that the enzyme possesses distinct sites for nucleotide and for carbamyl phosphate or carbamate and the mechanism of reaction with the substrate is of random type The results on the kinetic studies in the absence and presence of products also seem to be consistent with the Theorell-Chance mechanism [26] for which either MgdADP or carbamyl phosphate is the first substrate in the reverse reaction This possibility can, however, be ruled out since carbamyl phosphokinase has distinct binding sites for the nucleotide and for the carbamyl phosphate or carba-

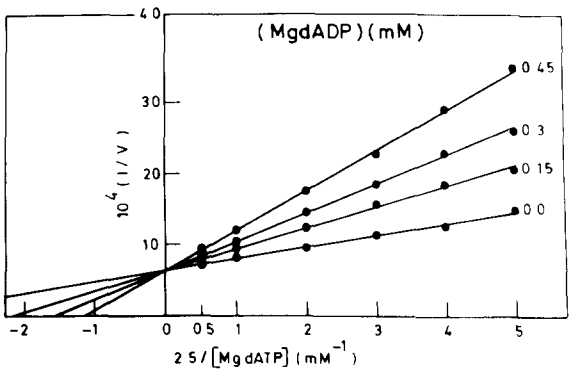

Fig 3 Competitive product inhibition of the forward reaction by MgdADP with MgdATP as the variable substrate with the concentration of carbamate held constant at 6 mM The concentrations of MgdADP are indicated on the lines of the double-reciprocal plot All the points indicated in the figure are the averages of three sets of experiments

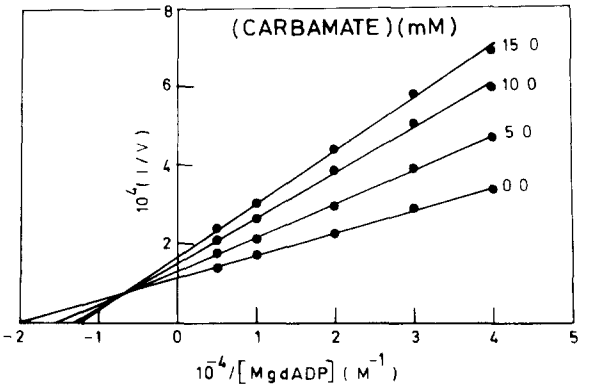

Fig 4 Non-competitive product inhibition of the reverse reaction by carbamate with MgdADP as the variable substrate and carbamyl phosphate held constant at 5 mM The concentrations of carbamate are indicated on the lines of double-reciprocal plot All the points shown in the figure are the averages of three sets of experiments

mate, which is not in accordance with the Theorell-Chance mechanism Marshall and Cohen [18] have suggested that the reaction of carbamyl phosphokinase with the substrate has an ordered mechanism

This mechanism however fails to explain their observation that carbamyl phosphate and adenine nucleotide can bind with the enzyme in the absence of each other, which is inconsistent with an ordered

TABLE III

INHIBITOR CONSTANTS FOR COMPETITIVE PRODUCT INHIBITION OF THE REVERSE AND FORWARD REACTIONS

Apparent inhibitor constants (K_I) are the averages ± S E of the values obtained from competitive inhibition data of three independent sets of experiments True K_I values were calculated from the apparent K_I values by the relationships described by Smith and Morrison [33] The concentrations of the fixed substrate were 5 mM carbamyl-phosphate, 0 2 mM MgdADP, 6 mM carbamate, 0 8 mM MgdATP

Inhibitor	Apparent K_I (mM)	K_I (mM)
A competitive inhibition of the reverse reaction		
1 by MgdATP with MgdADP	1 4 ± 0 13	1 02
2 by carbamate with carbamyl-phosphate	18 75 ± 1 0	15 22
B Competitive inhibition of the forward reaction		
1 by MgdADP with MgdATP	0 19 ± 0 007	0 10
2 by carbamyl-phosphate with carbamate	6 0 ± 0 62	2 13

TABLE IV

INHIBITOR CONSTANTS FOR NON-COMPETITIVE PRODUCT INHIBITION OF THE REVERSE AND FORWARD REACTIONS

Apparent inhibitor constants (K_I) are the averages ± S E of the values obtained from non-competitive inhibition data of three independent sets of experiments True K_I values were calculated from the apparent K_I values by the relationships as described by Smith and Morrison [33] The concentrations of the fixed substrate were 5 mM carbamyl-phosphate, 0 2 mM MgdADP, 6 mM carbamate, 0 8 mM MgdATP

Inhibitor	Apparent K_I (mM)	K_I (mM)
A Non-competitive inhibition of the reverse reaction		
1 by MgdATP with carbamylphosphate	0 24 ± 0 02	0 05
2 by carbamate with MgdADP	12 0 ± 1 52	3 42
B Non-competitive inhibition of the forward reaction		
1 by MgdADP with carbamate	0 2 ± 0 02	0 07
2 by carbamylphosphate with carbamate	5 5 ± 0 4	2 13

TABLE V

TEST OF KINETIC CONSTANTS FOR THEIR CONSISTENCY WITH THE HALDANE RELATIONSHIP

$K_1^{\text{Carbamyl-}P}$, K_1^{MgdATP}, K_1^{MgdADP} and $K_1^{\text{Carbamate}}$ are dissociation constants (or non-competitive inhibitor constants) of complexes of enzyme-MgdATP, enzyme-carbamylphosphate (carbonyl-*P*), enzyme-carbamate and enzyme-MgdADP with the products carbamyl-*P*, MgdATP, MgdADP and carbamate, respectively

Sl No		Relationship expected	Relationship observed
I	1	$K_s^{\text{MgADP}}K_m^{\text{Carbamyl-}P} = K_s^{\text{Carbamyl-}P}K_m^{\text{MgADP}}$	(0 06) (1 4) ≅ (2 0) (0 04)
	2	$K_s^{\text{MgdADP}}K_m^{\text{Carbamyl-}P} = K_s^{\text{Carbamyl-}P}K_m^{\text{MgdADP}}$	(0 14) (2 0) ≅ (4 7) (0 06)
	3	$K_s^{\text{MgGDP}}K_m^{\text{Carbamyl-}P} = K_s^{\text{Carbamyl-}P}K_m^{\text{MgGDP}}$	(0 25) (16 6) ≅ (3 4) (11 0)
	4	$K_s^{\text{MgCDP}}K_m^{\text{Carbamyl-}P} = K_s^{\text{Carbamyl-}P}K_m^{\text{MgCDP}}$	(2 6) (14 0) ≅ (2 0) (14 3)
	5	$K_s^{\text{MgUDP}}K_m^{\text{Carbamyl-}P} = K_s^{\text{Carbamyl-}P}K_m^{\text{MgUDP}}$	(4 3) (12 5) ≅ (4 0) (13 3)
II	1	$K_s^{\text{Carbamate}}K_m^{\text{MgATP}} = K_s^{\text{MgATP}}K_m^{\text{Carbamate}}$	(12 5) (0 6) ≅ (1 25) (6 0)
	2	$K_s^{\text{Carbamate}}K_m^{\text{MgdATP}} = K_s^{\text{MgdATP}}K_m^{\text{Carbamate}}$	(11 5) (0 5) ≅ (1 6) (3 7)
III		$K_i^{\text{Carbamyl-}P}K_s^{\text{MgdATP}} = K_s^{\text{Carbamyl-}P}K_i^{\text{MgdATP}}$	(2 13) (1 6) ≠ (4 7) (0 05)
IV		$K_1^{\text{MgdADP}}K_s^{\text{Carbamate}} = K_s^{\text{MgdADP}}K_1^{\text{Carbamate}}$	(0 07) (11 5) ≠ (0 14) (3 42)

mechanism Further, as shown in Table V, the numerical values of all kinetic constants were partially consistent with the Haldane relationship [27], which is characteristic of rapid equilibrium and random mechanism Isotope exchange studies would throw further light on this aspect

It is interesting to note that the substrate specificity, kinetic behaviour and reaction mechanisms of carbamyl phosphokinase and those of creatine kinase have some striking similarities with each other A comparison of kinetic constants of MgATP and MgADP and of carbamyl phosphate and carbamate could imply that, like creatine kinase, this enzyme may also be adapted for rapid phosphorylation of ADP in *S faecalis* and probably in other bacteria In this context, an interesting observation on the motility of fluorescent *Pseudomonas* sp and *Clostridium sporogenes* may be mentioned [28–30] Under anaerobic conditions, the lost motility of both the organisms can be restored by arginine supplementation which gives rise to rapid generation of ATP via carbamyl phosphokinase-catalyzed ADP + carbamyl phosphate reaction This gives suggestive evidence for the role of carbamyl phosphokinase in the mechanochemical operation of flagellar movement in *C sporogenes* and in *Pseudomonas* sp under anaerobic condition, somewhat resembling the role of creatine kinase in muscle contraction The similarities between the energy-dependent contractile mechanism of flagellar filaments of bacteria and that found in muscle have been noted by several authors [31] Though some of the *Streptococcus* sp belonging to Lancefield group D or enterococcus group are flagellated [32], it is generally believed (though not yet firmly demonstrated) that *S faecalis* is nonmotile and hence the suggested role of carbamyl phosphokinase in rapid phosphorylation of ADP may be linked to some other cellular events

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