Biochimica et Biophysica Acta, 370 (1974) 450–458
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BBA 67369

ON THE REGULATION AND ALLOSTERIC MODEL OF L-TYPE PYRUVATE KINASE FROM RAT LIVER

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(Received July 1st, 1974)

SUMMARY

1 The influence of P_i and the phosphorylated hexoses Fru-1,6-P_2, Fru-6-*P*, Fru-1-*P*, Glc-1,6-P_2, Glc-6-*P*, Glc-1-*P* and Gal-1-*P* on L-type pyruvate kinase from rat liver has been investigated at physiological ADP, *P*-enolpyruvate, GSH, ATP and alanine concentrations At 0 1 mM *P*-enolpyruvate and in the presence of 2 mM MgATP + 1 mM alanine the enzyme is inactive and the only effective activator in a physiological concentration range is Fru-1,6-P_2

2 The phosphorylated hexoses were used as a probe for the conformation of the enzyme A comparison of the effects of MgATP and alanine on the "affinities*" for the hexoses reveals the following differences (a) MgATP (2 mM) and alanine (1 mM) increase the concentration of Glc-1,6-P_2 and Glc-6-*P* necessary for half-maximal activation to the same extent, however, MgATP and alanine introduce a different cooperative interaction towards Glc-1,6-P_2 and Glc-6-*P* at low *P*-enolpyruvate concentrations (b) MgATP (2 mM) has less influence on the "affinities*" for Fru-1,6-P_2 and Fru-6-*P* as compared to alanine (1 mM) (c) Enzyme modification by oxidation of the thiol groups increases the effect of MgATP on the "affinity" for Glc-1,6-P_2, whereas the influence of alanine is decreased These results reveal the conclusion that MgATP and alanine introduce a different conformation, probably by binding at different sites The results are difficult to explain by the R $\rightleftharpoons$ T equilibrium model of Monod, Wyman and Changeux and indicate that sequential conformation changes are involved in the allosteric transitions of the enzyme

INTRODUCTION

Pyruvate kinase (ATP pyruvate phosphotransferase, EC 2 7 1 40) catalyses the last step of glycolysis Its activity is important in the regulation of the dynamic balance between gluconeogenesis and glycolysis in the liver In the liver two types of pyruvate kinase are present [1] The M_2-type is located in the non-parenchymal cells [2, 3] and although this type possesses regulatory properties [4–8], its presence in a cell seems to exclude gluconeogenesis The L-type is located in the parenchymal

* With "affinity" is meant the relative ability of a compound to activate the enzyme, this ability is concluded from the concentration of activator necessary for half maximal activation

cells [2, 3] and responds to diet and hormones [9] Although the L-type pyruvate kinase possesses many regulatory properties, the mechanism to inactivate the enzyme under gluconeogenic conditions is still uncertain [10–13] From the effect of the allosteric activators P_i, phosphorylated hexoses [10] and glucose-1,6-diphosphate (Glc-1,6-P_2) [11] on the L-type pyruvate kinase it was concluded that the presence of these compounds in rat liver will result in a fully active enzyme under both glycolytic and gluconeogenic conditions It was concluded that in the liver Glc-1,6-P_2, of which the concentration is quite constant, makes the regulation of the pyruvate kinase activity by Fru-1,6-P_2 impossible [11] However, these conclusions were based on experiments in which 0 5 mM *P*-enolpyruvate was used and in which effectors, such as alanine and ATP were absent Recent studies on pyruvate kinase from yeast [14] and erythrocytes [15] have shown that the affinity* of the enzyme for Fru-1,6-P_2 is increased by an increment in the *P*-enolpyruvate concentration This finding made it important to reinvestigate the affinity of pyruvate kinase for P_i and the phosphorylated hexoses at a more physiological *P*-enolpyruvate concentration (0 1 mM) to see to what extent the relatively high *P*-enolpyruvate concentration, which has been employed earlier, influences the measured affinities for these compounds The affinity of L-type pyruvate kinase for Fru-1,6-P_2 is also influenced by MgATP and alanine [12] For the reason that these compounds are both present in liver we investigated also the influence of P_i and the phosphorylated hexoses in the presence of the physiological concentrations of MgATP (2 mM) and alanine (1 mM) Under these conditions it can be concluded to what extent P_i and the phosphorylated hexoses contribute to the overall activity of pyruvate kinase in vivo

Our earlier studies with L-type pyruvate kinase from rat liver [16] and erythrocytes [17] have shown that the kinetic properties of the L-type are highly dependent upon the reduction state of the thiol groups, properties also found by Kutzbach and co-workers [13, 18] For this reason we isolated and assayed the enzyme in the presence of the physiological reductant reduced glutathione (GSH)

In the course of this investigation we became aware of the value of P_i and the phosphorylated hexoses as probes for the conformation of the enzyme The similarity in structure of these compounds and their similar kinetic behaviour [10, 11] has led to the conclusion that these compounds bind at the same activator site A change in conformation of this site by an effector will influence the affinity for P_i and the phosphorylated hexoses (this will be reflected in the concentration required for half maximal activation) and their way of binding (reflected in the Hill coefficient) In this way we can compare the effects of MgATP and alanine on the conformation of this activation site while also the effect of increasing *P*-enolpyruvate concentration can be determined

MATERIALS AND METHODS

Wistar rats, maintained on a normal diet and water ad libitum, were decapitated and the livers were removed Liver homogenates were prepared in 0 15 M NaCl containing 5 mM reduced glutathione (GSH) and centrifuged for 60 min at 100 000 × *g* From the supernatant, the partially purified L-type preparation was obtained according to the method of Passeron et al [19] The preparations were dissolved in 0 25 M Tris–HCl buffer (pH 7 5) containing 5 mM GSH The enzymatic activity was assayed

following the decrease in absorbance at 340 nm, at room temperature (22 °C) in a reaction mixture (3 0 ml) containing, unless indicated otherwise, 25 mM Tris–HCl buffer (pH 7 5), 0 1 mM KCl, 1 mM ADP, 0 12 mM NADH, 0 1 mg lactate dehydrogenase, 20 mM $MgCl_2$, 0 1 mM *P*-enolpyruvate and 5 mM GSH

The applied Mg^{2+} and K^+ concentrations are rather high, as compared to the free concentration found in liver [20] Therefore the reported experiments at 0 1 mM *P*-enolpyruvate were repeated at a free Mg^{2+} concentration of 1 mM (calculated by a method based on that of Morrison and co-workers [21, 22], which will be described in detail [37] The K^+ concentration was lowered from 100 to 25 mM These changes in ion concentrations did not change the obtained affinities for the phosphorylated hexoses

ADP, *P*-enolpyruvate, NADH, P_i and the phosphorylated hexoses were obtained from Boehringer (Mannheim, Germany) The phosphorylated hexoses were analysed for the presence of Fru-1,6-P_2 by the method of Bucher and Hohorst [23] Only Fru-6-*P* contained about 0 03% Fru-1,6-P_2, which contamination was removed enzymatically by the addition of aldolase, followed by deproteinization with trichloroacetic acid

Control assays were performed with P_i or the phosphorylated hexoses used, in the absence of *P*-enolpyruvate When MgATP or alanine or both were present, corresponding blanks were performed in the presence of these compounds In all cases there was no change of NADH absorption Duplicates of the reported experiments were run with twice the amount of lactate dehydrogenase to exclude possible effects on this enzyme reaction Doubling the amount of lactate dehydrogenase did not influence the results obtained

RESULTS

Affinity for P_i and the phosphorylated hexoses at 0 1 mM P-enolpyruvate and physiological effector concentrations

In preliminary studies on the effect of phosphorylated hexoses at 0 1 mM *P*-enolpyruvate, the L-type pyruvate kinase was isolated in the absence of a thiol group reductant The obtained affinity of the enzyme for Fru-1,6-P_2 was markedly decreased, as compared to the enzyme isolated in the presence of 1 mM mercaptoethanol (data not shown) The purpose of this study was to verify if the phosphorylated hexoses can influence the pyruvate kinase activity under physiological conditions Therefore we isolated the enzyme in the presence of the physiological reductant GSH (5 mM) [24]

Fig 1 shows the influence of Glc-1,6-P_2 at 0 1 mM *P*-enolpyruvate on the activity of L-type pyruvate kinase under various conditions At 0 1 mM *P*-enolpyruvate the concentration of Glc-1,6-P_2, necessary for half-maximal activation, is 120 μM In the presence of 2 mM MgATP or 1 mM alanine the Glc-1,6-P_2 concentration, necessary for half-maximal activation, is increased to about 300 μM, whereas in the presence of both alanine + MgATP the enzyme activity is hardly influenced by the applied Glc-1,6-P_2 concentrations Since the Glc-1,6-P_2 concentration in liver is about 15 μM [25], we can conclude that Glc-1,6-P_2 is not able to activate pyruvate kinase in the presence of the physiological concentrations of alanine and MgATP

Glc-6-*P* is somewhat less effective at a *P*-enolpyruvate concn of 0 1 mM as

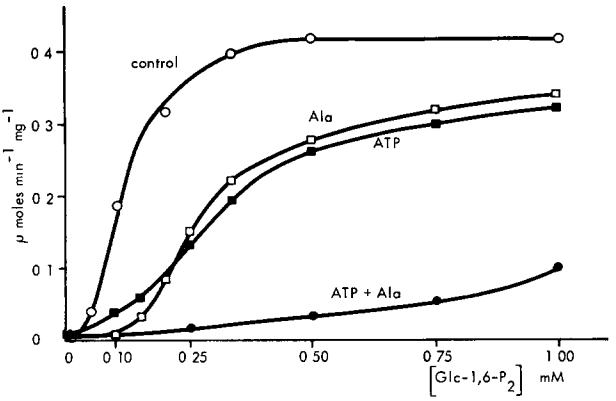

Fig 1 The activity vs Glc-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate concn = 0 1 mM, ADP concn = 1 mM and 5 mM GSH ○—○, control curve, □—□, in the presence of 1 mM alanine, ■—■, in the presence of 2 mM MgATP, ●—●, in the presence of 1 mM alanine + 2 mM MgATP

compared to Glc-1,6-P_2 (see Table I) The effects of 2 mM MgATP and 1 mM alanine on the affinity for Glc-6-*P* are comparable (figure not shown) In the presence of both inhibitors also the influence of Glc-6-*P* has almost disappeared, which seems to exclude a role of Glc-6-*P* in the regulation of pyruvate kinase [26]

Furthermore the following phosphorylated hexoses, Glc-1-*P*, Fru-1-*P*, Fru-6-*P*

TABLE I

THE AFFINITY OF TYPE-L-PYRUVATE KINASE FROM RAT LIVER FOR THE PHOSPHORYLATED HEXOSES AND P_i AT SEVERAL *P*-ENOLPYRUVATE CONCENTRATIONS

The values are the concentrations of hexoses (μM) and P_i (mM) necessary for half maximal activation The corresponding Hill values are calculated The *P*-enolpyruvate concentrations used are indicated

	Control	*n* Value	+alanine (1 mM)	*n* value	+MgATP (2 mM)	*n* value	Alanine (1 mM) + MgATP (2 mM)	*n* value
P-Enolpyruvate concn = 0 1 mM								
Fru-1,6-P_2	1 2	2 6	3 2	2 6	1 8	2 8	4 5	3 9
Fru-6-*P*	No effect		No effect		No effect		No effect	
Glc-1,6-P_2	120	2 7	290	3 3	310	2 0	~ 1000	
Glc-6-*P*	190	2 7	590	2 4	590	1 9	~1500	
P_i	No effect		No effect		No effect		No effect	
P-Enolpyruvate concn = 0 25 mM								
Fru-1,6-P_2	1 2	2 1	3 6	2 9	2 0	2 8	5 0	3 0
Fru-6-*P*	>1500		No effect		No effect		No effect	
Glc-1,6-P_2	70	2 0	210	2 9	230	2 1	600	3 0
Glc-6-*P*	70	2 1	200	2 9	230	2 2	600	3 0
P_i	>30		No effect		No effect		No effect	
P-Enolpyruvate concn = 0 50 mM								
Fru-1,6-P_2	0 4	1 1	2 1	1 9	1 5	2 3	4 5	2 3
Fru-6-*P*	900		No effect		1500		No effect	
Glc-1,6-P_2	50	1 6	170	2 2	170	2 1	500	3 1
Glc-6-*P*	50	1 5	170	1 9	170	2 0	500	3 2
P_i	16	3 2	~30		>30		No effect	

and Gal-1-*P* were tested in a concentration range between 0 and 1 5 mM and for P_i between 0 and 30 mM Under the applied conditions (identical as for Figs 1 and 2) these compounds did not influence the pyruvate kinase activity, even in the absence of MgATP or alanine These obtained data, at 0 1 mM *P*-enolpyruvate and physiological effector concentrations, make it unlikely that P_i and the phosphorylated hexoses (except Fru-1,6-P_2) are able to influence the pyruvate kinase activity in vivo

Fig 2 shows that under the applied conditions the only activator in a physiological concentration range will be Fru-1,6-P_2 In accordance with other reports [12,

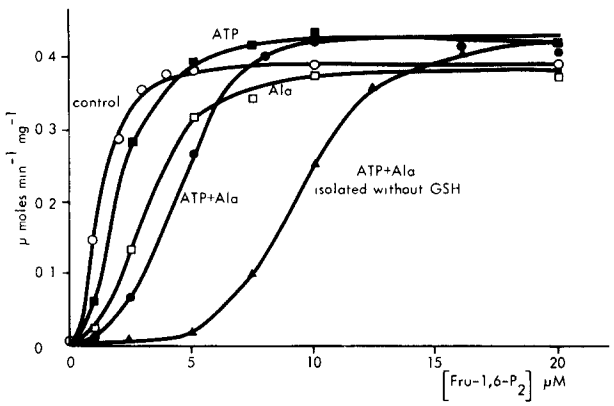

Fig 2 The activity vs Fru-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate = 0 1 mM, ADP concn = 1 mM and 5 mM GSH ○—○, control curve, □—□, in the presence of 1 mM alanine, ■—■, in the presence of 2 mM MgATP, ●—●, in the presence of 1 mM alanine + 2 mM MgATP, ▲—▲, in the presence of 1 mM alanine + 2 mM MgATP, the enzyme was isolated and assayed in the absence of GSH

13] it can be observed that the affinity of the enzyme for Fru-1,6-P_2 is high even in the presence of both MgATP and alanine This figure shows also that the affinity for Fru-1,6-P_2 of the enzyme, isolated in the presence of GSH, is more influenced by 1 mM alanine as compared with 2 mM MgATP This figure shows further that the enzyme isolated in the absence of the physiological reductant has a markedly lower affinity for Fru-1,6-P_2 and is practically inactive at 5 μM Fru-1,6-P_2

The phosphorylated hexoses and P_i as a probe for the conformation of the enzyme

As already mentioned in the Introduction, the phosphorylated hexoses and P_i can be used as a probe for the conformation of the activator site From the similarity of the structure of these compounds and from data reported elsewhere [10, 11] it can be concluded that these compounds bind on the same activator site The use of these compounds has the advantage over fluorescent dyes that their influence is measured as enzymatic activity, which excludes a non-specific reaction Furthermore, the affinity for these compounds can be measured accurately, which is an advantage over the relative insensitivity of the fluorescent probe methods [28]

Table I shows the *n* values calculated from the Hill plots (not shown) and the corresponding concentrations of phosphorylated hexoses necessary for half-maximal activation At a *P*-enolpyruvate concentration of 0 1 mM the enzyme has a higher

affinity for Glc-1,6-P_2 than for Glc-6-*P*, both in the absence and presence of MgATP and/or alanine From the obtained *n* values we can conclude that the activators bind in a cooperative way 1 mM alanine or 2 mM MgATP increase the Glc-1,6-P_2 concentration necessary for half-maximal activation to the same extent However, the *n* value in the presence of alanine is significantly higher as in the presence of MgATP which means that the conformational state of the enzyme in the presence of alanine differs from that with MgATP The same phenomenon is observed with Glc-6-*P* as activator, whereas with Fru-1,6-P_2 as activator no significant difference is observed

At 0 25 mM *P*-enolpyruvate the difference in affinity between Glc-1,6-P_2 and Glc-6-*P* has disappeared (middle of Table I) This indicates that by increasing the *P*-enolpyruvate concentration not only the affinity for Glc-1,6-P_2 and Glc-6-*P* increases, but that there is also a change in specificity The activator site can no longer discriminate between Glc-1,6-P_2 and Glc-6-*P* Such behaviour cannot be explained by a simple shift of the R $\rightleftharpoons$ T equilibrium to the R state and indicate that *P*-enolpyruvate changes the conformation of the activator site in such a way that the phosphate on the C_1 place of the glucose molecule does no longer influence the effector binding At 0 25 mM *P*-enolpyruvate the cooperativity toward Fru-1,6-P_2, Glc-1,6-P_2 and Glc-6-*P* is decreased as compared with 0 10 mM *P*-enolpyruvate Also at this *P*-enolpyruvate concentration the cooperativity for Glc-1,6-P_2 and Glc-6-*P* in the presence of alanine is higher than in the presence of MgATP

A further increase in *P*-enolpyruvate concentration to 0 5 mM results in a higher affinity for the phosphorylated hexoses and P_i The binding of Fru-1,6-P_2 is no longer cooperative, while the cooperativity towards Glc-1,6-P_2 and Glc-6-*P* is further decreased The cooperativity in the presence of alanine is also decreased, while this does not occur in the presence of MgATP This leads to a disappearance of the difference in *n* values observed at 0 25 and 0 1 mM *P*-enolpyruvate A comparison of the effects of MgATP and alanine on the affinity for the phosphorylated hexoses reveals the following differences (a) 2 mM MgATP increases the concentration of Glc-1,6-P_2 and Glc-6-*P* necessary for half-maximal activation to the same extent as 1 mM alanine, the influence of MgATP on the affinity for Fru-1,6-P_2 and Fru-6-*P*, however, is less when compared to the influence of alanine (b) Although MgATP and alanine increase the concentrations of Glc-1,6-P_2 and Glc-6-*P*, necessary for half-maximal activation to the same extent, they introduce a different cooperative interaction towards these activators at 0 25 and 0 10 mM *P*-enolpyruvate These differences indicate that MgATP and alanine introduce a different conformation, probably by binding to different sites The evidence for binding to different sites is presented in Fig 3 In this figure is plotted the influence of Glc-1,6-P_2 on the enzyme, isolated in the absence of a thiol group reductant The influence of alanine on the affinity for Glc-1,6-P_2 is decreased, as compared to Fig 1 (half maximal activation at 190 μM instead of 290 μM), whereas the effect of MgATP on the contrary is increased Incubation of this enzyme with 5 mM GSH at 10 °C for 1 h converts the enzyme into a form with the same kinetic properties as reported in Fig 1, suggesting that the modification is the consequence of oxidation of thiol groups This difference in response to modification of the enzyme strengthens the earlier statement that MgATP and alanine bind to different allosteric sites

These results can also explain the discrepancies described in literature between the sensitivities for MgATP and alanine [12, 29] of L-type pyruvate kinase, which

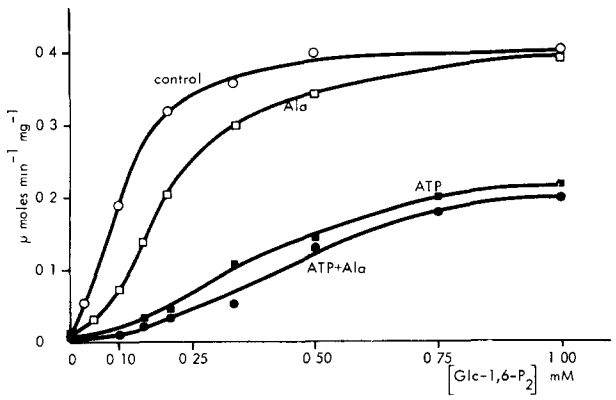

Fig 3 The activity vs Glc-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate = 0 1 mM and ADP concn = 1 mM The enzyme was isolated and assayed in the absence of GSH ○—○, control curve, □—□, in the presence of 1 mM alanine, ■—■, in the presence of 2 mM MgATP, ●—●, in the presence of 1 mM alanine + 2 mM MgATP

has been extensively discussed by Seubert and Schoner [30] in a recent review Schoner et al [29] purified the enzyme in the absence of a thiol group reductant and found the enzyme more sensitive to MgATP than to alanine, while the opposite was found by Llorente et al [12] with crude enzyme extracts It seems likely that in the enzyme, as isolated by Schoner et al [29], thiol groups are oxidized during the isolation procedure, which would explain their kinetic properties (cf Fig 3) With the enzyme, isolated according to Llorente et al [12], such a modification would not occur

DISCUSSION

The influence of P_i and the phosphorylated hexoses on L-type pyruvate kinase from rat liver has led to the conclusion that the presence of these compounds in liver results in an active enzyme under both glycolytic and gluconeogenic conditions [10,11] Our data show that the affinity of L-type pyruvate kinase for P_i and the phosphorylated hexoses is increased by raising the *P*-enolpyruvate concentration The affinities for P_i and the phosphorylated hexoses were tested earlier [10, 11] at 0 5 mM *P*-enolpyruvate Lowering of the *P*-enolpyruvate concentration to an in vivo value [32] results in lower affinities for P_i and the phosphorylated hexoses, which are further decreased, when the inhibitors MgATP and/or alanine are also present From the affinities, obtained at physiological concentrations of *P*-enolpyruvate, MgATP and alanine it can be concluded that P_i and the phosphorylated hexoses (except Fru-1,6-P_2) in their physiological concentration range [25, 26] will not be able to activate pyruvate kinase These findings reduce the number of possible regulatory legends to a great extent

The extrapolation of in vitro data to the in vivo regulation of an enzyme needs some assumptions However, it has been shown for the L-type from yeast, which is very similar to the L-type from rat liver, that its properties in situ are comparable to data obtained in vitro [32, 33] For reason that the L-type pyruvate kinase is inactive in the presence of physiological concentrations of *P*-enolpyruvate, MgATP and

alanine it seems reasonable to conclude that in the liver cell the enzymatic activity will be dependent upon the Fru-1,6-P_2 concentration However, as a consequence of the high affinity for Fru-1,6-P_2, the enzyme will be active even in the presence of the lowest reported Fru-1,6-P_2 concentration for liver For this reason Sols and Marco [27] proposed that under fasting conditions the free Fru-1,6-P_2 concentration in liver will be lower than 5 μM due to binding to aldolase To our opinion two other additional possibilities exist

(A) A change in the enzyme itself can influence its affinity for Fru-1,6-P_2 as is shown in Fig 2 and cf [13] and [16] Since feeding influences the GSH/GSSG ratio in liver (10 under feeding and 4 under fasting conditions, unpublished), the enzyme was isolated in the presence of the various ratio's of GSH and GSSG However, the affinity for Fru-1,6-P_2 was found to be equal to the affinity obtained for the enzyme isolated in the presence of 5 mM GSH alone Further investigations are therefore required to see if such an enzyme modification can occur within the cell

(B) This possibility is based on the heterogeneity of the liver 35% of the liver cells is of non-parenchymal origin These cells contribute to about 10% of the liver mass [34] Under glycolytic conditions it is reasonable to assume that Fru-1,6-P_2 is equally divided between the different cell types The Fru-1,6-P_2 concentration under this condition is 30 μM, in which the non-parenchymal cells will contribute 3 nmoles/g and the parenchymal cells 27 nmoles/g Under fasting conditions, gluconeogenesis is introduced in the parenchymal cells and not in the non-parenchymal cells [2, 3] The Fru-1,6-P_2 concentration under these conditions is 5 μM of which 3 nmoles/g are located in the non-parenchymal cells and the other 2 nmoles/g are contributed by the parenchymal cells Since these cells occupy 90% of the liver mass, the Fru-1,6-P_2 concentration in these cells will be 2 2 μM How much the above mentioned three possibilities mutually contribute to the overall regulation of pyruvate kinase by Fru-1,6-P_2, cannot be said and deserve more experimentation in which isolated parenchymal cells may be a useful object

The use of phosphorylated hexoses as a probe for the conformation of the enzyme makes it clear that the two-state model of Monod et al [35] cannot explain our results with the phosphorylated hexoses From the different behaviour of the enzyme towards the phosphorylated hexoses in the presence of ATP or alanine we can conclude that these inhibitors induce a different conformation The different behaviour towards modification suggests that these different conformations are the consequence of binding at different allosteric centers Recently Rozengurt et al [36] showed that K^+ and Fru-1,6-P_2 introduce different conformation states rather than a unique R state as predicted by the model of Monod et al [35] Our results obtained in the presence of MgATP and alanine show that these inhibitors introduce a different T conformation and suggest that sequential conformation changes [28] are involved in the allosteric transitions of the enzyme

ACKNOWLEDGEMENTS

Mrs A A E d'Hamecourt-Broekmans and Miss A C Hanson are thanked for the preparation of the manuscript The Netherlands Foundation for Fundamental Medical Research (FUNGO) is acknowledged for partial financial support (Grant 13-39-05)

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