Biochimica et Biophysica Acta, 571 (1979) 45—54
© Elsevier/North-Holland Biomedical Press

BBA 68851

KINETICS AND EQUILIBRIA OF PYRIMIDINE NUCLEOSIDE MONOPHOSPHATE KINASE FROM HUMAN ERYTHROCYTES

EDWARD M. SCOTT and RITA C. WRIGHT

Alaska Investigation Division, Center for Disease Control, 225 Eagle Street, Anchorage, AK 99501 (U.S.A.)

(Received May 3rd, 1979)

Key words: Pyrimidine nucleoside monophosphate kinase; (Human erythrocyte)

Summary

The common type of pyrimidine nucleoside monophosphate kinase (ATP:CMP phosphotransferase, EC 2.7.4.14), purified 50 000-fold from human erythrocytes, reacted with a wide variety of nucleotides, but only ATP, dATP, UMP and CMP were good substrates. The optimum Mg^{2+} concentration, 2—3 mM, was generally independent of substrate concentration, of the nature of the substrate, and of the direction of the reaction. Kinetic studies indicated that a ternary complex was formed, that the substrates were bound at two unlike sites, and that the order of addition of substrates was random. Equilibrium constants were ATP + UMP 0.98, ATP + CMP 1.59, dATP + UMP 1.13, and ATP + AMP 1.20.

Introduction

The pyrimidine nucleoside monophosphate kinase (ATP:CMP phosphotransferase, EC 2.7.4.14) of human erythrocytes became of genetic interest when it was shown to be polymorphic in human populations [1]. Formation of the enzyme is controlled by three allelic genes, and the six phenotypes that can result are readily differentiated [2].

Two genetically different forms of the enzyme have been purified and compared [3]. Cheng and Domin [4] investigated the separation of the enzyme from other kinases in erythrocytes. Similar mammalian enzymes have been prepared from calf liver [5], calf thymus [6], and rat liver [7]. The substrate specificity of these enzymes was similar in that they catalyzed the phosphorylation of UMP, CMP, or dCMP by ATP or dATP but the reverse reaction and

detailed kinetics were not studied. To investigate these aspects, we have purified the common form (form 1) of the enzyme from human erythrocytes.

Methods

Enzyme assay. 1 ml of a reaction mixture containing 0.1 M Tris-HCl (pH 8.0), 0.1 M KCl, 2 mM $MgCl_2$, 2 mM ATP, 1.5 mM phospho*enol*pyruvate, 0.14 mM NADH, 13 mM 2-mercaptoethanol, 3 units pyruvate kinase (from rabbit muscle), 1 unit lactate dehydrogenase (from beef heart), and pyrimidine nucleoside monophosphate kinase was equilibrated at 30° C for 10 min to allow ADP, an impurity in ATP, to react. Then UMP was added to give a concentration of 2 mM, and the rate of decrease in absorbance at 340 nm was determined. The rate was corrected for a blank with no UMP. Since the reaction is coupled, a steady-state rather then an initial rate was determined. A unit of activity was taken as the amount of enzyme that produced 1 μmol UDP/min and is equivalent to 2 μmol NADH oxidized/min, since both ADP and UDP are substrates of pyruvate kinase.

Protein concentration was determined from absorbance at 280 nm, and specific activity was expressed in units/mg protein.

Reaction rates. For kinetic studies in the forward direction, the above assay mixture was used with various substrate concentrations. With other substrates additional pyruvate kinase was added when this enzyme was found to limit the rate. dCDP did not react with pyruvate kinase and dUDP reacted at a negligible rate.

The mixture for the reverse reaction contained the same concentrations of buffer, KCl, $MgCl_2$ and 2-mercaptoethanol used above plus 10 mM glucose, 1 mM NADP, 0.4 unit hexokinase (from yeast), 0.4 unit glucose-6-phosphate dehydrogenase (from yeast), pyrimidine nucleoside monophosphate kinase, and the desired concentrations of purine and pyrimidine nucleoside diphosphates in a volume of 1 ml. Increase in absorbance at 340 nm and 30° C was measured, and the rate was calculated as μmol NADPH formed/min. With some substrates additional hexokinase was required. The rate of reaction of dGTP with hexokinase was very slow. All reaction rates were steady-state rates and are expressed as $\mu mol \cdot min^{-1} \cdot unit^{-1}$ of enzyme.

Equilibrium constants. The reaction mixture contained the same concentrations of buffer, KCl, $MgCl_2$ and 2-mercaptoethanol used in the assay plus nucleoside phosphates as indicated and an excess of the enzyme (about 0.1 unit). After incubation at 30° C for 2 h, the enzyme was inactivated by addition of 0.1 vol. 10 M HCl. The solution was adjusted to pH 8.0 with 10 M NaOH, centrifuged, and aliquots were used as the substrate in the assays below.

UDP plus ADP and UMP. An aliquot of the equilibrium mixture was the sole substrate in the assay in the forward direction but without the enzyme; the sum of the concentrations of UDP and ADP was calculated from the net decrease in absorbance at 340 nm. Then an excess of the enzyme was added, and the concentration of UMP calculated from the additional net decrease in absorbance. ATP cycled in the latter system and we did not find it necessary to add an excess. The final products were UTP and ATP.

ATP and UDP. An aliquot of the equilibrium mixture was the sole substrate under the conditions used for the reverse reaction but without the enzyme. ATP concentration was calculated from the net increase in absorbance at 340 nm. Then an excess of the enzyme and more ADP (0.2 mM) were added, and UDP concentration was calculated from the additional net increase in absorbance. The final products were ADP and UMP.

The millimolar change in absorbance upon reduction was taken to be 6.2 for both NAD and NADP. Concentration of ADP was calculated by difference. Deoxyadenosine phosphates and cytidine phosphates were determined in the same way.

Purification. Human erythrocytes (outdated cells from a blood bank) were washed with buffered NaCl solution (one part 0.1 M phosphate, pH 7.4, and nine parts 0.9% NaCl) and frozen. To 1 l cells were added 4 l water and 100 g DEAE-cellulose. (The DEAE-cellulose had been washed twice with 2-l portions of 0.01 M phosphate, pH 7.4, and was added as a moist cake.) After being stirred for 15 min, the mixture was poured into a Buchner funnel with a coarse sintered-glass bottom and filtered by suction. The filter cake was washed with 1 l 0.01 M phosphate, pH 7.4, and the enzyme eluted with 1 l 0.1 M phosphate, pH 5.5, containing 0.2 M NaCl and 1 mM $MgCl_2$. Fractions of 100 ml were collected, and those containing the enzyme were combined.

To each 100 ml of eluate were added 35 g $(NH_4)_2SO_4$; the precipitate was removed by centrifugation and discarded. An additional 14 g $(NH_4)_2SO_4$/100 ml were added to the solution, and the precipitate was removed by centrifugation and dissolved in 10 ml 0.01 M histidine-HCl, pH 6.5, containing 1 mM EDTA and 13 mM 2-mercaptoethanol.

This solution was passed through a 5 × 75 cm column of polyacrylamide gel (P-100). The active fractions were combined and dialyzed for 8 h against a solution containing 0.1 M histidine-HCl, pH 6.5, 30% glycerol, 1 mM EDTA, and 13 mM 2-mercaptoethanol (buffer A).

A 2 × 10 cm column was filled with Affigel Blue (Agarose coupled with the dye, Reactive Blue 2). After the column was washed with buffer A, the dialyzed enzyme was added, followed by 100 ml buffer A, 100 ml buffer A containing 0.5% $(NH_4)_2SO_4$, and 100 ml buffer A containing 1% $(NH_4)_2SO_4$. The fractions with specific activities above 60 were combined and concentrated by ultrafiltration.

Concentrated solutions from three such preparations were combined and the adsorption step with the dye-agarose column was repeated. The active eluate was concentrated by ultrafiltration and stored at −20°C.

Results

Purification and stability

The purification summarized in Table I gave a 5% yield of enzyme with more than a 50 000-fold increase in specific activity. The enzyme activity found in erythrocytes may vary two-fold in people of the common phenotype (form 1), and a higher specific activity of the purified enzyme was attained if the cells that were used contained at least 1.8 units of the enzyme/ml. The electrophoretic mobility of the purified enzyme on starch gel was the same as that

TABLE I

PURIFICATION ON PYRIMIDINE MONOPHOSPHATE KINASE

Treatment	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)
Hemolyzate	5000	1960	$35 \cdot 10^4$	0.0056
DEAE-cellulose	302	1080	3600	0.30
$(NH_4)_2SO_4$	16	385	980	0.39
Polyacrylamide gel	50	266	46	5.8
Dye-agarose gel	47	170	1.9	90
Dye-agarose gel	0.8	101	0.30	337

found in hemolyzates from people of phenotype form 1. Nucleoside diphosphokinase, adenosine triphosphatase, and guanosine monophosphate kinase could not be detected in the purified enzyme, but small amounts (less than 0.3%) of adenylate kinase were present in some preparations. When necessary, an appropriate blank was used to correct for adenylate kinase.

The purified enzyme was notably unstable. In the absence of glycerol, the half-life of a preparation with a specific activity of 80 was about 10 min at 20°C. Inactivation was partially reversed by addition of 2-mercaptoethanol. The enzyme was more stable in histidine buffer than in phosphate and more stable at pH 6.5 than at higher pH. If the concentrated purified enzyme was stored in 30% glycerol at −20°C, loss of activity was less than 5%/month. In kinetic studies frequent standardization was used to correct for instability.

Substrate specificity

Relative rates of reaction with various substrates are shown in Tables II and III. In testing for possible substrates, sufficient enzyme was used to detect 0.02% of the activity found with ATP + UMP. TDP, GDP, IDP, XDP, dADP

TABLE II

RELATIVE RATES OF REACTION OF THE ENZYME WITH VARIOUS SUBSTRATES

Forward reaction			Reverse reaction		
Substrate (0.25 mM)	UMP (0.25 mM)	CMP (0.1 mM)	Substrate (0.25 mM)	UDP (0.25 mM)	CDP (0.25 mM)
ATP	100	100	ADP	100	100
dATP	90	86	dADP	124	54
ITP	0.3	0.5	IDP	8	6
dITP	0.2	0.4	*		
GTP	0.3	0.2	GDP	0.03	0
dGTP	0.2	0.3	dGDP	0	0
XTP	0.04	0.2	XDP	0	0.07
UTP	1.0	1.6	UDP	0.14	0
CTP	0.1	0.3	CDP	0	0.3
dUTP	0.02	0.4	dUDP	0	0
dCTP	0.5	1.9	dCDP	0	0
TTP	0	0	TDP	0	0

* dIDP was not available to us.

TABLE III

KINETIC CONSTANTS OF PYRIMIDINE NUCLEOSIDE MONOPHOSPHATE KINASE

Substrates	Forward reaction						Reverse reaction					
	V (μmol · min^{-1} unit^{-1})	K_u (mM)	K_y (mM)	K_{uy} ((mM)^2)	K_{uy}/K_u (mM)	K_{uy}/K_y (mM)	V' (μmol · min^{-1} unit^{-1})	K'_u (mM)	K'_y (mM)	K'_{uy} ((mM)^2)	K'_{uy}/K'_u (mM)	K'_{uy}/K'_y (mM)
ATP, UMP	1.08	0.08	0.18	0.018	0.22	0.10	0.55	0.04	0.22	0.007	0.16	0.03
ATP, CMP	0.71	0.010	0.07	0.003	0.31	0.04	0.37	0.011	0.06	0.0016	0.15	0.03
ATP, dCMP	0.36	0.09	0.71	0.09	1.0	0.12	0.10	0.10	0.50	0.017	0.17	0.03
ATP, dUMP	0.16	0.05	2.7	0.39	7.3	0.14	0.02	0.018	0.43	0.032	1.7	0.07
dATP, UMP	0.94	0.10	0.05	0.008	0.08	0.16	1.00	0.10	0.38	0.011	0.11	0.03
dATP, CMP	0.33	0.003	0.015	0.003	1.0	0.23	0.23	0.03	0.08	0.0006	0.02	0.01
dATP, dCMP	0.56	0.26	0.33	0.14	0.54	0.42	0.25	0.32	1.45	0.045	0.14	0.03
dATP, dUMP	*						0.03	0.40	1.0	0.06	0.15	0.06
ITP, UMP	0.25	3.5	8.5	3.6	1.0	0.4	0.15	0.58	0.04	0.25	0.43	6.3
dITP, UMP	0.25	1.5	16	5.5	3.7	0.3	*					
GTP, UMP	0.04	0.11	1.2	1.8	16	1.5	*					

* Kinetic studies of these substrates were prevented by technical difficulties.

and dGDP did not react with ADP, and TMP and IMP did not react with ATP. The reaction catalyzed is

Purine nucleoside triphosphate + pyrimidine nucleoside monophosphate ⇋
purine nucleoside diphosphate + pyrimidine nucleoside diphosphate

UTP and CTP could replace ATP to a limited extent. Only thymidine nucleotides were unreactive, but with several substrate combinations only the forward reaction could be demonstrated.

Kinetics

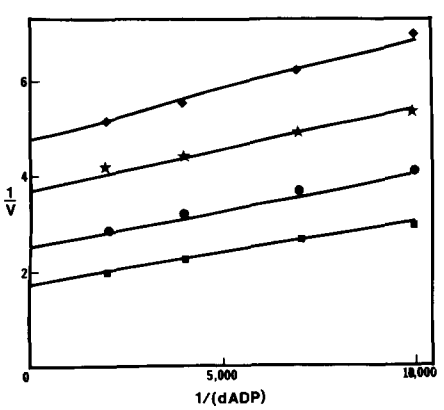
The steady-state rates of reaction (v) were fitted to the equation

$$1/v = 1/V + K_u/V[U] + K_y/V[Y] + K_{uy}/V[U][Y] \quad (1)$$

V is a maximum rate, K_u, K_y and K_{uy} are constants, [U] is the concentration of purine nucleoside triphosphate, and [Y] is the concentration of pyrimidine nucleoside monophosphate. In the reverse direction the comparable constants are denoted by primes. The results presented in Table III were obtained by calculating steady-state rates with four concentrations of one substrate at each of four concentrations of the other. The concentrations used were between 0.025 and 0.5 mM. A typical example is shown in Fig. 1.

Substrate and product inhibition

Some substrates were inhibitory at high concentrations, and this inhibition was competitive with the second substrate. If the inhibiting substrate was a purine nucleotide, the substrate inhibition constant (K_{is}) was calculated from



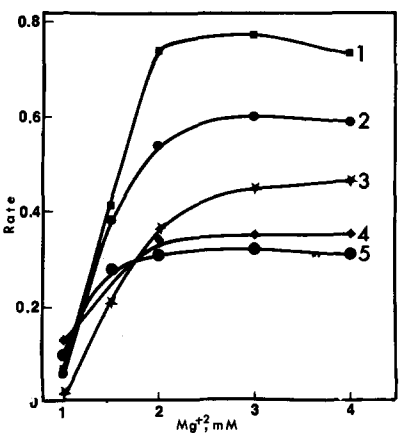


Fig. 1. Effect of substrate concentration in the dADP + UDP reaction. Reaction rate (v) is in μmol · min^{-1} · unit^{-1} of enzyme. The lines were drawn from the constants from Table III and represent UDP concentrations from top to bottom of 0.1, 0.14, 0.25 and 0.5 mM.

Fig. 2. Effect of Mg^{2+} concentration on reaction rate, expressed in μmol · min^{-1} · unit^{-1} of enzyme at 30°C and pH 8.0. The substrates were: 1, 0.5 mM ATP + 0.5 mM UMP; 2, 0.25 mM ATP + 0.25 mM UMP; 3, 0.25 mM dATP + 0.25 mM UMP: 4, 0.5 mM ADP + 0.5 mM UDP; 5, 0.25 mM ATP + 0.25 mM CMP.

the equation

$$1/v = 1/V + K_u/V[U] + (K_y/V[Y] + K_{uy}/V[U][Y])(1 + [U]/K_{is})$$

The calculated values of K_{is} were ATP 1.5 mM, dATP 0.2 mM, CMP 0.05 mM, and CDP 0.5 mM. Other substrates were not inhibitory.

For technical reasons, the only product that could be used for inhibition studies was UMP. With ADP and UDP as substrates, the product inhibition constants K_{ip} and K'_{ip} were calculated from the equation

$$1/v' = 1/V' + K'_u/V'[U] + (K'_y/V'[Y])(1 + [I]/K_{ip}) + (K'_{uy}/V'[U][Y])(1 + [I]/K'_{ip})$$

[I] was UMP concentration. For UMP, K_{ip} was 0.25 mM and K'_{ip} was 0.15 mM.

Mg^{2+} concentration and pH

The optimal concentration of Mg^{2+} for both the forward and reverse reactions was determined with ATP, dATP, UMP, and CMP at several different substrate concentrations. The optimum, 2—3 mM, was generally independent of substrate concentration and of the nature of the substrates, although minor differences were found. Typical results are shown in Fig. 2. For kinetic studies, an excess of Mg^{2+} (2 mM) was used. From known dissociation constants of Mg^{2+} and adenine nucleotides [8], over 90% of nucleoside triphosphate, about 60% of nucleoside diphosphate, and 8% of nucleoside monophosphate would be expected to be in the form of magnesium complexes under these conditions. Activity increased moderately with pH between pH 7 and 9 as shown in Fig. 3. Outside this range, technical difficulties were encountered.

Equilibria

A determination of the equilibrium constant

$$K_{eq} = \frac{[ADP][UDP]}{[ATP][UMP]}$$

is shown in Table IV. Since the substrates were not pure, the concentrations of

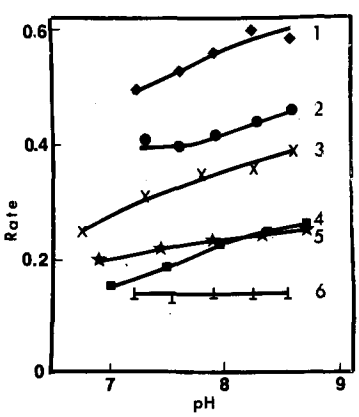


Fig. 3. Effect of pH on reaction rate expressed in $\mu mol \cdot min^{-1} \cdot unit^{-1}$ of enzyme at 30° C. The concentration of all substrates was 0.25 mM. The substrates were: 1, ATP + UMP; 2, dATP + UMP; 3, ADP + UDP; 4, dADP + UDP; 5, ADP + CDP; 6, ATP + CMP.

TABLE IV

EQUILIBRIUM CONSTANT

pH 8.0, 30°C, Mg^{2+} 2 mM.

Starting substrates	Final concentration (mM)				K_{eq}
	ATP	UMP	ADP	UDP	
ATP, UMP	0.52	0.53	0.53	0.47	0.90
	0.67	0.19	0.41	0.29	0.93
	0.17	0.70	0.36	0.32	0.97
	0.26	0.27	0.31	0.23	1.02
ADP, UDP	0.53	0.55	0.61	0.49	1.03
	0.33	0.34	0.68	0.15	0.91
	0.31	0.36	0.20	0.57	1.02
	0.23	0.24	0.26	0.22	1.04
				Mean 0.98 ± 0.02	

the products at equilibrium were not necessarily equal to the amounts formed in the reaction.

Equilibrium constants for other systems were dATP + UMP 1.13 ± 0.02, ATP + CMP 1.59 ± 0.09, and ATP + AMP 1.20 ± 0.06. The last value was determined with adenylate kinase from rabbit muscle at pH 8.0 and 30°C and with a Mg^{2+} concentration equal to half the total concentration of adenosine phosphates. The equilibrium constant in the case of ATP + CMP was less reliable than the other because the pyruvate kinase used contained a small amount of cytidine monophosphate kinase activity.

Discussion

Although a variety of nucleoside phosphates are substrates of pyrimidine nucleoside monophosphate kinase, only those of adenosine, deoxyadenosine, uridine and cytidine reacted rapidly. At substrate concentrations of 0.25 mM, ATP reacted with dCMP at 12%, and with dUMP at 2%, of the rate found with UMP. The forward reaction was usually faster than the reverse reaction, but IDP reacted with UDP or CDP at a much higher rate than ITP reacted with UMP or CMP. UTP did not react with IMP.

The minor activities of the enzyme cannot be ignored completely. If the system were in true equilibrium, ATP and UMP would not only form UDP and ADP, but UDP would also form UTP. (The presence of nucleoside diphosphokinase would give the same result.) In the method used here, UTP would be measured as ATP, and the equilibrium constant would be calculated to be half its true value. Starting with ATP and UMP, the amount of UDP formed would be less than the amount of ADP formed provided, however, that adenylate kinase was not present. If adenylate kinase were present, formation of AMP would lower the concentrations of both ATP and ADP. AMP would not be measured and the presence of adenylate kinase would not affect the equilibrium constant of the ATP/UMP system. In practice, the effect of minor activities of the enzyme was minimized by appropriate controls, and no con-

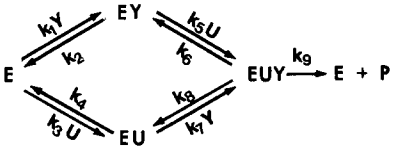


Fig. 4. Random sequential reaction scheme for pyrimidine nucleoside monophosphate kinase.

vincing evidence was obtained of interference with the reactions of rapidly reacting substrates.

The kinetic results are consistent with the formation of a ternary complex involving two unlike binding sites on the enzyme. These sites differ in that one of them binds pyrimidine nucleoside mono- or diphosphate, while the other binds purine nucleotides preferentially. The two sites are not equivalent since AMP will not react with UTP nor will ADP and UDP form AMP and UTP. Also, the inhibition by UMP of the ADP + UDP reaction was competitive with UDP but not with ADP.

Competitive substrate inhibition appeared to result from binding at an improper site, such as the binding of a purine nucleotide to a site that normally bound pyrimidine nucleotides. Since one cannot easily determine whether a substrate binds at the improper site more or less readily if it is also bound at the proper site, the substrate inhibition constants were derived on the arbitrary assumption that binding at the improper site was not affected by binding at the proper site.

The order of binding of the substrates appeared to be random. Thus, if it were obligatory that the purine nucleotide bind first, K_u/V should equal K_1 in Fig. 4. If it were obligatory that ATP bind first, one would then expect K_u/V for ATP to be the same regardless of the other substrate, but K_u/V varied 20-fold for both ATP and dATP. Similarly, K_y/V for UMP, K'_u/V' for ADP and dADP, and K'_y/V' for UDP were variable depending on the other substrate.

When the order of binding of substrates is random, Eqn. 1 is customarily derived by assuming that the binding of each substrate to the free enzyme is rapid enough to be in equilibrium. If this assumption is correct, one would expect K_{uy}/K_y (k_4/k_3 in Fig. 4) to be the same for ATP (or dATP) regardless of the second substrate and this seems to be true. K'_{uy}/K'_y for ADP and for dADP are also relatively constant. One would also expect K_{uy}/K_u (k_2/k_1 in Fig. 4) to be the same for UMP with any other substrate, but this was not the case and it seems possible, therefore, that the binding of at least one substrate with the free enzyme is not in equilibrium.

The equilibrium constant depends on the difference in free energy of a terminal triphosphate bond and a terminal diphosphate bond. This difference would be expected to be small and would not differ greatly with different nucleotides; it varied between —220 and 14 cal · mol^{-1} in the examples studied. Strominger et al. [5] reported the equilibrium constant of the ATP + CMP system to be 1.01 and that of the adenylate kinase system to be 1.21. Markland and Wadkins [9] found a value of 1.23 for the latter.

The physiologic function of the enzyme is unknown, but some of its effects can be predicted. From Fig. 3, it should be active at physiologic pH. In the erythrocyte the predominant nucleotides are ATP (1.3 mM), ADP (0.1 mM),

and AMP (0.01 mM) [10]. The high level of ATP is maintained by glycolysis, and the low level of AMP by adenylate kinase, which is present in about ten times the amount found for the enzyme. In the presence of the enzyme and nucleoside diphosphokinase, the nucleotides of uridine and cytidine would tend to be phosphorylated to the same extent as the adenosine nucleotides.

References

1 Giblett, E.R., Anderson, J.E., Chen, S.-H., Teng, Y.-S. and Cohen, F. (1974) Am. J. Hum. Genet. 26, 627—635
2 Scott, E.M. and Wright, R.C. (1978) Ann. Hum. Genet. 41, 341—346
3 Teng, Y.-S., Chen, S.-H. and Scott, R.C. (1976) J. Biol. Chem. 251, 4179—4183
4 Cheng, Y.-C. and Domin, B. (1978) Anal. Biochem. 85, 425—429
5 Strominger, J.L., Heppel, L.A. and Maxwell, E.S. (1959) Biochim. Biophys. Acta 32, 412—421
6 Sugino, Y., Teraoka, H. and Shimono, H. (1966) J. Biol. Chem. 241, 961—969
7 Maness, P. and Orengo, A. (1975) Biochemistry 14, 1484—1489
8 Alberty, R.A. (1969) J. Biol. Chem. 244, 3290—3302
9 Markland, F.S. and Wadkins, C.L. (1966) J. Biol. Chem. 241, 4136—4143
10 Dean, B.M., Perrett, D. and Sensi, M. (1978) Biochem. Biophys. Res. Commun. 80, 147—154