BBA 65983

UDP-GLUCOSE:GLYCOGEN α-4-GLUCOSYLTRANSFERASE I KINASE ACTIVITY OF PURIFIED MUSCLE PROTEIN KINASE

CYCLIC NUCLEOTIDE SPECIFICITY

K. K. SCHLENDER*, S. H. WEI AND C. VILLAR-PALASI**

Department of Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minn. 55455 (U.S.A.)

(Received June 2nd, 1969)

SUMMARY

A purified protein kinase obtained from rabbit skeletal muscle catalyzed the conversion of transferase I to transferase D. The same enzyme preparation also activated phosphorylase *b* kinase (phosphorylase *b* kinase kinase activity) and catalyzed the phosphorylation of casein. Under the conditions employed, the transferase I kinase activity was essentially dependent upon cyclic AMP. The K_A value of cyclic AMP for transferase I kinase activity was 70 nM. The activation was quite specific for cyclic AMP. Changing the base moiety (cyclic GMP, cyclic CMP or cyclic UMP) increased the K_A value 60–100-fold. The binding was even more adversely affected by changes in the ribose moiety (cyclic dAMP and cyclic TMP). The maximum activation for all of the 3′,5′-cyclic nucleotides studied varied only about 20%. A 3′,5′-cyclic phosphate group appeared to be a requirement for activation as 2′,3′-cyclic AMP, 3′-AMP and 5′-AMP were without activity.

INTRODUCTION

In skeletal muscle, the I form of UDP-glucose:glycogen α-4-glucosyltransferase (transferase EC 2.4.1.11) is converted into the D form by a phosphorylation catalyzed by a protein kinase which requires ATP and Mg^{2+} (ref. 1). Using relatively crude preparations obtained from skeletal muscle which contained both transferase I and transferase I kinase activity, it was shown that cyclic AMP stimulated the ATP–Mg^{2+}-dependent conversion of transferase I to transferase D[2–6].

Changes in the levels of transferase I *in vivo* obtained with several hormone treatments have been attributed to alterations in transferase I kinase[7] or in the degree of activation of the kinase reaction by altered levels of cyclic AMP[8]. Other cyclic

Abbreviations: 3′,5′-cyclic mononucleotides, *i.e.* cyclic AMP, adenosine 3′,5′-cyclic phosphate.

* Present address: Department of Pharmacology, Medical College of Ohio at Toledo, Toledo, Ohio 43614, U.S.A.

** Present address: Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Va. 22903, U.S.A.

nucleotides, for example the naturally occurring cyclic GMP, may also affect the transferase I kinase reaction[6]. Thus, it seemed important to study: (a) the transferase I kinase reaction using purified enzyme preparations free of interfering enzymes, (b) the quantitative dependence on cyclic AMP of this kinase-catalyzed reaction and (c) the specificity of this activation by various cyclic nucleotides and cyclic nucleotide analogs. The results of these studies are presented in this paper.

EXPERIMENTAL DETAILS

Assay of transferase I kinase

Transferase I kinase activity was assayed by measuring the kinase-catalyzed decrease in transferase I upon incubation with ATP–Mg^{2+}. The transferase I substrate was purified from rabbit skeletal muscle. Fresh rabbit muscle was extracted by homogenizing 1:3 (w/v) with 50 mM Tris–HCl and 5 mM EDTA buffer (pH 7.7). After centrifugation, the supernatant was precipitated with 30% ethanol (−5°) and the precipitate resuspended in 50 mM Tris–HCl, 5 mM EDTA and 50 mM mercaptoethanol buffer (pH 6.9). The dissolved preparation was incubated at 4° until essentially all of the transferase was converted to the I form (usually 6–8 h). Insoluble material was removed by centrifugation and the supernatant was applied to a DEAE-cellulose column. The enzyme was eluted from the column as previously reported[9] and the enzyme precipitated with 15% ethanol. The precipitated enzyme was dissolved in 50 mM Tris-HCl and 5 mM EDTA buffer (pH 7.7) and stored in small aliquots at −60°. Transferase I was purified to a specific activity of about 5 units/mg (1 unit = 1 μmole of glucose incorporated into glycogen per min at 30°) and had a transferase I/total ratio of 0.95. The details of this purification are to be published elsewhere[10]. Transferase I was diluted to 0.5 units/ml with a solution of 50 mM Tris–HCl buffer, 5 mM EDTA, 5 mg gelatin per ml, 10 mM dithiothreitol (pH 7.7) and preincubated for 30 min at 30°. The transferase I kinase reaction mixture contained 25 mM Tris–HCl buffer, 2.8 mM dithiothreitol, 1.4 mg gelatin per ml, about 0.125 unit transferase I per ml, 5 mM ATP, 10 mM $MgCl_2$, 5 mM EDTA, and 2.5 mM theophylline in a total volume of 180 μl; the pH of the reaction mixture was 7.4. When added, cyclic AMP was included at a concentration of 50 μM. Kinase activity was adjusted to use no more than 25% of the transferase I during the reaction. The reaction mixtures were incubated at 30°, and at 0 and 5 min, aliquots of 30 μl were mixed with 60 μl of standard transferase I reaction mixture[11] modified to contain 20 mM EDTA, 25 mM KF, and 3 mM Na_2SO_4. The excess EDTA prevented any further kinase activity. The Na_2SO_4 was included to activate transferase I (ref. 12). Transferase I activity was determined as previously described[11]. The activity of transferase I kinase is expressed as a decrease in transferase I ($-\Delta$ munits per 5 min) during the kinase incubation.

Protein kinase preparation

The early stages of kinase purification were similar to those used by WALSH *et al.*[13]. The protein which was eluted from the first DEAE-cellulose column[13] by 30 mM potassium phosphate buffer was further purified. The column eluate which was obtained from 525 g of fresh rabbit muscle was equilibrated with 5 mM Tris-HCl and 2 mM EDTA buffer (pH 7.5) by chromatography on Sephadex G-25. The enzyme was absorbed on a Whatman DE-52 DEAE-cellulose column (1 cm × 15 cm) which was

equilibrated with the same buffer. The column was washed with one bed volume of the equilibrating buffer. The bulk of the transferase I kinase activity was eluted with 20 mM Tris-HCl and 2 mM EDTA buffer (pH 7.5). The enzyme was purified approx. 300-fold. (It was difficult to accurately determine transferase I kinase activity in the crude homogenate.) This protein kinase preparation also catalyzed the phosphorylation of casein and the activation of phosphorylase *b* kinase when assayed as previously described[13]. The kinase fraction which contained 1–2 mg protein per ml was stable for at least 4 months when stored at −60°.

Nucleotides

Cyclic GMP, cyclic UMP, cyclic TMP, and cyclic dAMP were synthesized as described by SMITH *et al.*[14]. Other nucleotides were purchased from Sigma Chemical Co., St Louis, Mo.

RESULTS

Transferase I kinase activity of the protein kinase

When the purified kinase was incubated with transferase I and ATP–Mg^{2+}, transferase I was converted into transferase D (Table I). The reaction was essentially

TABLE I

TRANSFERASE I KINASE ACTIVITY OF PURIFIED PROTEIN KINASE IN THE ABSENCE AND PRESENCE OF CYCLIC AMP

Protein kinase (50 μg/ml) was incubated with transferase I and ATP–Mg^{2+} as described in the text. The activity of the enzyme is expressed as a decrease in the munits of transferase I as compared to the o-time control. There was no change in transferase I if the protein kinase or ATP–Mg^{2+} was omitted from the assay mixture.

Compound	*Concn. (μM)*	*Kinase activity (−Δ munits transferase I per 5 min)*
None	—	0.020
Cyclic AMP	50	0.575

dependent upon cyclic AMP when assayed under the conditions described above. There was no conversion of transferase I to transferase D when the kinase was omitted from the reaction mixture. However, each transferase I preparation had to be assayed for kinase activity since it has been found that an occasional preparation may have significant kinase activity. It was also demonstrated that during the transferase I to transferase D conversion, there was concomitant cyclic AMP-dependent transfer of ^{32}P from [γ-^{32}P]ATP to the protein fraction. There were 9.9 nmoles of $^{32}P_i$ incorporated per unit of the I form converted to the D form. This was in good agreement with the value of 9.5 nmoles per unit recently reported by LARNER *et al.*[15]. The conversion of transferase I to transferase D as the kinase concentration was varied is shown in Fig. 1. The reaction was proportional to kinase concentration when less than 25% of the transferase I substrate was utilized. The reaction was also linear with time until

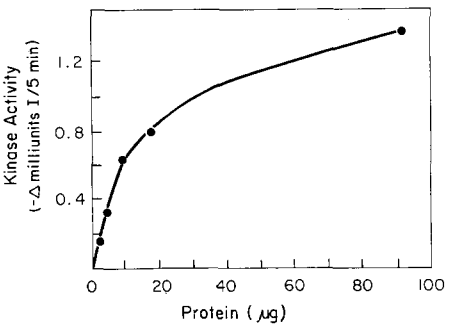

Fig. 1. Transferase I kinase activity at varying concentrations of protein kinase. Protein was determined by the method of LOWRY *et al.*[22]. Kinase assay as described in text. Cyclic AMP concentration was 50 μM.

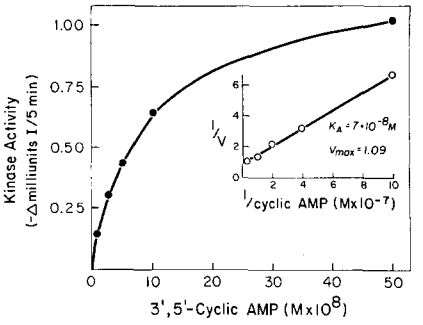

Fig. 2. Transferase I kinase activity at varying concentrations of cyclic AMP. Protein kinase was included at 50 μg/ml. Assay as described in text.

about 20–25% of the transferase I was converted. In Fig. 2, transferase I kinase activity at varying concentrations of cyclic AMP is given. From the $1/v$ *vs.* $1/[S]$ double reciprocal plot, the concentration for half-maximal activation (K_A) was determined to be about 70 nM.

Specificity of cyclic nucleotide activation

The specificity of the cyclic nucleotide activation of transferase I kinase activity is shown in Table II. The apparent affinity of the activators was changed considerably when the base moiety of the molecule was varied. Cyclic GMP, cyclic CMP, and cyclic UMP had K_A values which ranged from 60- to 100-fold higher than cyclic AMP. The binding of cyclic nucleotide was even more sensitive to changes in the ribose moiety. Cyclic dAMP, where the only change from cyclic AMP was the removal of a hydroxyl group on the 2'-position, required a 1500-fold higher concentration for half-maximal activation of the kinase activity. With cyclic TMP, where there is both a change in the base and the 2'-position of the ribose, a concentration of 1.3 mM (about 20 000-fold greater than cyclic AMP) was required for half-maximal activation.

TABLE II

SPECIFICITY OF CYCLIC NUCLEOTIDE ACTIVATION OF TRANSFERASE I KINASE ACTIVITY

Assay as described in text.

Compound	K_A *(μM)*	v_{max} *(−Δ munits transferase I per 5 min)*
Cyclic AMP	0.067	0.962
Cyclic GMP	9.9	0.869
Cyclic CMP	8.9	0.807
Cyclic UMP	6.5	0.792
Cyclic dAMP	110	0.843
Cyclic TMP	1300	0.854
3'-AMP	—	—
5'-AMP	—	—
2',3'-cyclic AMP	—	—

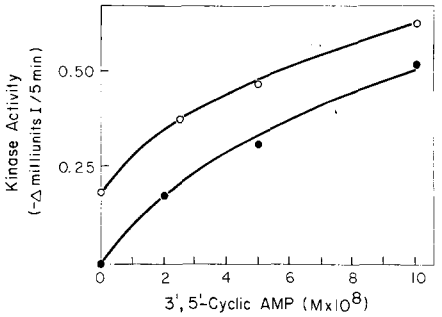

Fig. 3. Transferase I kinase activity with varying concentrations of cyclic AMP in the absence (●) or presence (○) of 2 μM cyclic GMP. The concentration of protein kinase was 50 μg/ml. Assay as described in text.

On the other hand, maximal activation was relatively insensitive to changes in structure (Table II). The lowest maximum stimulation of the kinase reaction was obtained with cyclic UMP. When included at saturating concentration, cyclic UMP was 80% as effective as cyclic AMP.

Cyclic GMP has been identified as a normal component of urine[16] and animal tissues[17]. The K_A for cyclic GMP activation of transferase I kinase (Table II) was found to be considerably higher than the levels of cyclic GMP reported by GOLDBERG *et al.*[17]. To further investigate the possible role of cyclic GMP in the control of transferase I kinase, we determined if the combination of cyclic GMP and cyclic AMP resulted in additive or antagonistic effects. Transferase I kinase activity was determined while varying the concentration of cyclic AMP in the presence and absence of nonsaturating levels of cyclic GMP (2 μM). As shown in Fig. 3, the stimulatory effects produced by cyclic GMP and cyclic AMP were found to be additive.

The presence of the 3',5'-cyclic phosphate group on the nucleotide seemed to be an absolute requirement for activation of transferase I kinase. Thus, 3'-AMP, 5'-AMP, and 2',3'-cyclic AMP had no effect at concentrations as high as 2.5 mM (Table III).

TABLE III

EFFECT OF ADENINE MONONUCLEOTIDES ON CYCLIC AMP ACTIVATION OF TRANSFERASE I KINASE ACTIVITY

The concentration of cyclic AMP was 0.5 μM. Assay as described in text.

Compound	*Concn. (mM)*	*Kinase activity (−Δmunits transferase I per 5 min)*
None	—	0.957
2',3'-cyclic AMP	0.05	0.956
	2.5	0.980
3'-AMP	0.05	0.987
	2.5	0.932
5'-AMP	0.05	0.859
	2.5	0.914

KULKA AND STERNLICHT[18] have reported that 3'-AMP competitively inhibited cyclic AMP-stimulated release of amylase by the pancreas. They have suggested that 3'-AMP inhibition can be used to detect the participation of cyclic AMP in other reactions. Accordingly, we determined the effect of 3'-AMP as well as 5'-AMP and 2',3'-cyclic AMP on the cyclic-AMP-dependent activity of transferase I kinase. For this study, cyclic AMP was included at 0.5 μM. As presented in Table III, the adenine mononucleotides did not inhibit transferase I kinase activity even at a concentration 5000-fold greater than cyclic AMP.

DISCUSSION

The purified protein kinase preparation exhibited transferase I kinase activity as well as the previously reported activities towards protamine, casein, and phosphorylase *b* kinase[13]. The enzyme was totally dependent upon cyclic AMP for activity under the specified assay conditions. The K_A for cyclic AMP with transferase I as a substrate was about 70 nM. This was in good agreement with the value of 60 nM reported by HUIJING AND LARNER[4] for the stimulation of a relatively crude preparation containing transferase I and transferase I kinase activities. The reported K_A values of cyclic AMP for the phosphorylation of casein and protamine by the purified protein kinase were 0.1 μM and 60 nM respectively[13]. The K_A value for cyclic AMP activation of the protein kinase with phosphorylase *b* kinase as the substrate has not been reported. The level of cyclic AMP required for half-maximal activation of phosphorylase *b* kinase presumably contaminated with the protein kinase was 70 nM (ref. 19).

To establish if all of the kinase activities are associated with one general protein kinase or if there are separate specific kinases in the same fraction will require further study. Epinephrine increases cyclic AMP levels in skeletal muscle[8], affects *in vivo* levels of transferase I[20], and also activates phosphorylase *b* kinase[21]. Therefore, it will be of particular interest to determine if transferase I kinase and phosphorylase *b* kinase kinase are the same or different enzymes.

The activation of transferase I kinase activity was quite specific for cyclic AMP. Changes in the base moiety increased the apparent binding constant of the activator. The binding constant of the activator was even more sensitive to changes of the ribose moiety, *i.e.*, the K_A for cyclic dAMP was over 1000-fold greater than that of cyclic AMP. The maximal activation was not greatly different for any of the 3',5'-cyclic nucleotides tested. There appeared to be an absolute requirement for a 3',5'-cyclic phosphate group. Mononucleotides which did not contain the 3',5'-cyclic phosphate group did not activate the enzyme, and they did not compete with cyclic AMP activation.

Cyclic GMP which is a naturally occurring nucleotide was an effective activator of the enzyme. However, while the tissue levels are lower[17], the level of cyclic GMP required for transferase I kinase activation is about 70-fold higher than the cyclic AMP requirement. The activation by cyclic AMP and cyclic GMP were additive. Thus, it appears doubtful that cyclic GMP is important for the control of this transferase I kinase activity *in vivo*.

ACKNOWLEDGMENTS

The authors are grateful to Professor J. Larner for his helpful advice and discussions. This investigation was supported by the U.S. Public Health Service Research Grant AM-09071. K.K.S. was a postdoctoral fellow of the United States Public Health Service.

REFERENCES

1 D. L. FRIEDMAN AND J. LARNER, *Biochemistry*, 2 (1963) 669.
2 M. ROSELL-PEREZ AND J. LARNER, *Biochemistry*, 3 (1964) 81.
3 M. M. APPLEMAN, E. BELOCOPITOW AND H. N. TORRES, *Biochem. Biophys. Res. Commun.*, 14 (1964) 550.
4 F. HUIJING AND J. LARNER, *Biochem. Biophys. Res. Commun.*, 23 (1966) 259.
5 F. HUIJING AND J. LARNER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 647.
6 O. WALAAS, E. WALAAS AND S. OSAKI, in W. J. WHELAN, *Control of Glycogen Metabolism*, Universitetsforlaget, Oslo, 1968, p. 139.
7 C. VILLAR-PALASI AND J. I. WENGER, *Federation Proc.*, 26 (1967) 563.
8 N. D. GOLDBERG, C. VILLAR-PALASI, H. SASKO AND J. LARNER, *Biochim. Biophys. Acta*, 148 (1967) 665.
9 C. VILLAR-PALASI, M. ROSELL-PEREZ, S. HIZUKURI, F. HUIJING AND J. LARNER, *Methods Enzymol.*, 8 (1966) 374.
10 K. K. SCHLENDER AND J. LARNER, in preparation.
11 J. A. THOMAS, K. K. SCHLENDER AND J. LARNER, *Anal. Biochem.*, 25 (1968) 486.
12 J. A. THOMAS, K. K. SCHLENDER AND J. LARNER, in preparation.
13 D. A. WALSH, J. P. PERKINS AND E. G. KREBS, *J. Biol. Chem.*, 243 (1968) 3763.
14 M. SMITH, G. I. DRUMMOND AND H. G. KHORANA, *J. Am. Chem. Soc.*, 83 (1968) 698.
15 J. LARNER, C. VILLAR-PALASI AND N. E. BROWN, *Biochim. Biophys. Acta*, 178 (1969) 470.
16 T. D. PRICE, D. F. ASHMAN AND M. M. MELICOW, *Biochim. Biophys. Acta*, 138 (1967) 452.
17 N. D. GOLDBERG, S. DIETZ AND A. O'TOOLE, *J. Biol. Chem.*, in the press.
18 R. G. KULKA AND E. STERNLICHT, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1123.
19 R. J. DELANGE, R. G. KEMP, W. D. RILEY, R. A. COOPER AND E. G. KREBS, *J. Biol. Chem.*, 243 (1968) 2200.
20 B. J. WILLIAMS AND S. E. MAYER, *Mol. Pharmacol.*, 2 (1966) 454.
21 J. B. POSNER, R. STERN AND E. G. KREBS, *J. Biol. Chem.*, 240 (1965) 982.
22 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.