Volume 309, number 3, 275–278 FEBS 11514 September 1992

p56^{lyn} catalyzes a reversible autophosphorylation reaction and a nucleoside diphosphate kinase reaction

Craig M.E. Litwin, Marthe Gendreau and Jerry H. Wang

MRC Group in Signal Transduction, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada

Received 27 July 1992

The reversible autophosphorylation of the pp60$^{c\text{-}src}$ family tyrosine kinase, p56^{lyn} has been characterized by a simple procedure that involves the examination of the enzyme catalyzed radioisotope exchange between ATP and ADP. The equilibrium constant of the reaction was determined to be 3.31 and corresponded to a standard free energy of hydrolysis of the phosphotyrosine bond in p56^{lyn} of −8.08 kcal/mol. GDP was capable of substituting for ADP as phosphate acceptor so that p56^{lyn} displayed a nucleoside diphosphate kinase activity.

Tyrosine kinase; p56^{lyn} autophosphorylation; Nucleoside diphosphate kinase

1. INTRODUCTION

A number of tyrosine kinases have been shown to undergo a reversible phosphorylation reaction readily, including pp60$^{c/v\text{-}src}$ [1,2], the abl tyrosine kinase [3], and the EGF receptor [4]. In contrast, serine/threonine kinases do not appear to effectively catalyze a reversible phosphorylation reaction [5]. The underlying biochemical mechanism and physiological relevance of a readily reversible phosphorylation reaction at present remains undefined, but hints at fundamental mechanistic differences between serine/threonine phosphorylation reactions compared to tyrosine phosphorylation reactions.

We have previously reported the purification of the pp60$^{c\text{-}src}$ related tyrosine kinase p56^{lyn} from bovine spleen [6]. Common to pp60$^{c\text{-}src}$ related tyrosine kinases, p56^{lyn} undergoes autophosphorylation upon incubation with ATP. This autophosphorylation could not be blocked by pre-incubation with unlabelled ATP suggesting that p56^{lyn} may also catalyze a readily reversible autophosphorylation reaction. In the present study, a procedure has been established for the demonstration and characterization of the autophosphorylation reaction of p56^{lyn}. In addition, it has been documented that p56^{lyn} possesses a nucleoside diphosphate kinase activity that effectively catalyses the phosphorylation of GDP to GTP.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (4500 Ci/mmol) was obtained from ICN. Tritiated nucleotides were obtained from Amersham. Glycine, HEPES, SDS, and Tris were obtained from Bohringer Mannheim. Nonidet P-40 (NP 40) was obtained from Fluka Biochemicals. All other chemicals were purchased from Sigma. p56^{lyn} was purified as previously reported except that the Red A-agarose step was omitted [6].

Correspondence address: J.H. Wang, MRC Group in Signal Transduction, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, T2N 4N1, Canada.

2.2. *Reversal of p56^{lyn} phosphorylation by ADP*

An aliquot of p56^{lyn} was incubated in the presence of 50 mM Tris-HCl pH 7, 25 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP with or without 500 μM ADP at 30°C for the indicated times. At 30 min, ADP was added to one set of samples to initiate the reverse reaction. An equal volume of buffer was added to control samples. To stop the reaction, an aliquot of the reaction mixture was removed and mixed with SDS sample buffer [8]. The samples were resolved by SDS-PAGE and p56^{lyn} located by autoradiography. The radioactive bands were excised from the gel and counted by liquid scintillation spectroscopy.

2.3. *Phosphorylation of [^{3}H]purine dinucleotides to [^{3}H]purine trinucleotides by p56^{lyn}*

p56^{lyn} was incubated in the presence of 50 mM Tris-HCl pH 7, 25 mM $MgCl_2$, 100 μM ATP and 200 nM [^{3}H]ADP or [^{3}H]GTP (16 Ci/mmol) at 30°C for the indicated time. The reaction was stopped and analyzed by HPLC. Adenosine nucleotides were analyzed isocratically by HPLC using a Waters 5 μ C_{18} Resolve column (3.9 mm × 150 mm) equilibrated with 2% acetonitrile and 1% H_3PO_4 pH 6.0 adjusted with triethanolamine, at a flow rate of 2 ml/min. Up to 20 μl of sample was injected per run. Guanosine nucleotides were analyzed in a similar manner except that the HPLC buffer did not contain acetonitrile. Samples were quantitated with a Flow-Beta in line radioactivity detector. Linearity of response of the detector was determined by calculation of peak areas relative to known quantities of injected radioactivity. Counting efficiency was determined by calibration using a known quantity of radioactivity.

3. RESULTS AND DISCUSSION

3.1. *Demonstration of the reversible autophosphorylation reaction of p56^{lyn}*

The reversible autophosphorylation reaction can be represented by the equation: $E + ATP \rightleftharpoons E_p + ADP$ (1)

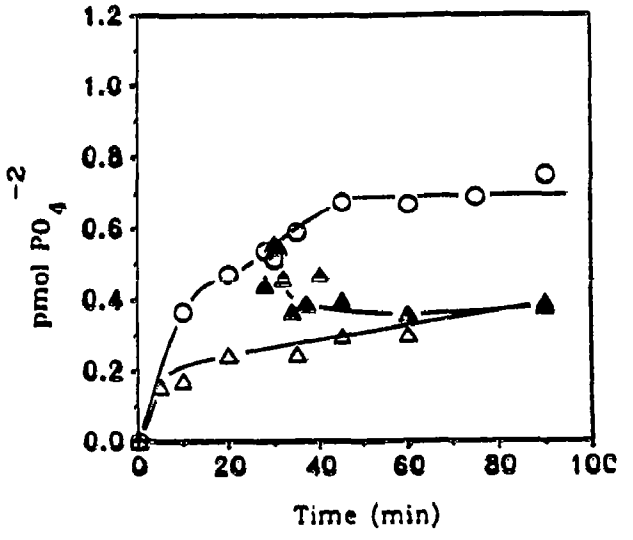

Fig. 1. ADP induced reversal of $p56^{lyn}$ autophosphorylation. Three separate aliquots of $p56^{lyn}$ were incubated under autophosphorylation conditions as described in section 2 with (▲), or without (○) 500 μM ADP. A small volume of 37.7 mM ADP was added to one set (▲) to a final concentration of 500 μM. Equal volume aliquots were removed from each sample at the indicated time and analyzed by SDS-PAGE.

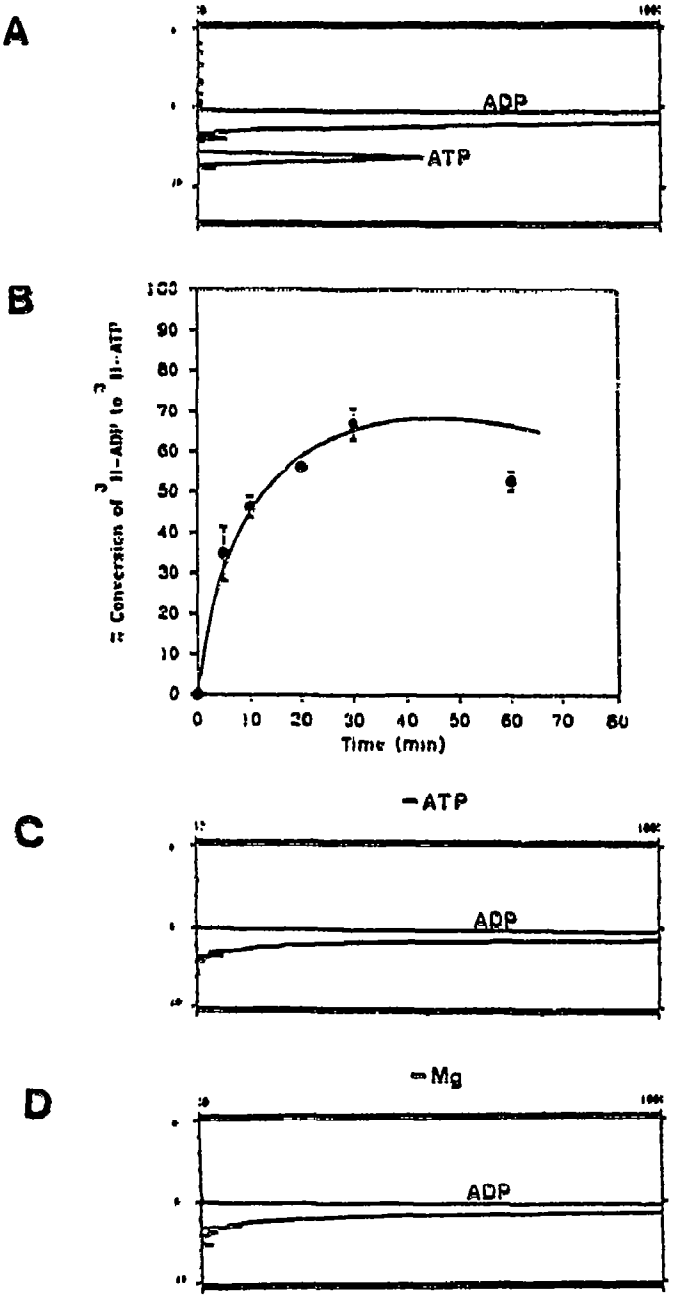

Fig. 2. $p56^{lyn}$ catalyzed conversion of [^{3}H]ADP to [^{3}H]ATP. (A) HPLC resolution of [^{3}H]ADP and [^{3}H]ATP. (B) Time-course of [^{3}H]ATP formation catalyzed by $p56^{lyn}$. (C) Analysis of reaction incubated for 60 min in the absence of ATP. (D) Analysis of the reaction incubated for 60 min in the absence of $MgCl_2$. An aliquot of $p56^{lyn}$ was incubated in the presence of 50 mM Tris-HCl pH 7, 25 mM $MgCl_2$, 100 μM ATP, and 200 nM [^{3}H]ADP at 30°C. At the indicated times, the reaction was stopped by the addition to a final concentration of 5 mM ADP, 5 mM ATP and 50 mM EDTA and analyzed by HPLC as outlined in section 2.

where E represents unphosphorylated kinase and E_p represents phosphorylated kinase. Equation (1) predicted that incubation of the phosphoenzyme in the presence of ADP should favour the reverse reaction and initiate the apparent dephosphorylation of the phosphokinase. Commensurate with the apparent dephosphorylation of $p56^{lyn}$ would be the phosphorylation of ADP to ATP. As shown in Fig. 1, incubation of $p56^{lyn}$ in the presence of [γ-^{32}P]ATP resulted in the incorporation of $^{32}PO_4^{-2}$ into the protein. When a 5-fold excess of ADP was included during this incubation, the extent of autophosphorylation was substantially reduced. If $p56^{lyn}$ was first allowed to autophosphorylate for 30 min at which time a 5-fold excess of ADP was added, radioactivity bound to the enzyme would undergo a rapid decrease to levels comparable to ADP control values (Fig. 1).

The loss of radioactive phosphate from $p56^{lyn}$ could be due either to a phosphatase reaction or to reversible autophosphorylation. In previous studies, the definitive demonstration of a reversible phosphorylation was carried out utilizing prelabelled kinase and following the transfer of the protein bound phosphate [1–4]. While viable, this approach requires extensive manipulation to isolate a usable quantity of labelled kinase with high specific radioactivity. In addition, the kinase so isolated must still retain activity. A much simpler procedure, however, can be used to descriminate between a reversible autophosphorylation and a phosphatase reaction. According to Equation (1), a reversible autophosphorylation reaction should lead to an exchange between ADP and ATP. Thus, a conversion of [^{3}H]ADP to [^{3}H]ATP would be expected in the kinase autophosphorylation reaction.

To analyze the conversion of [^{3}H]ADP to [^{3}H]ATP, a novel HPLC protocol was developed. Fig. 2A shows

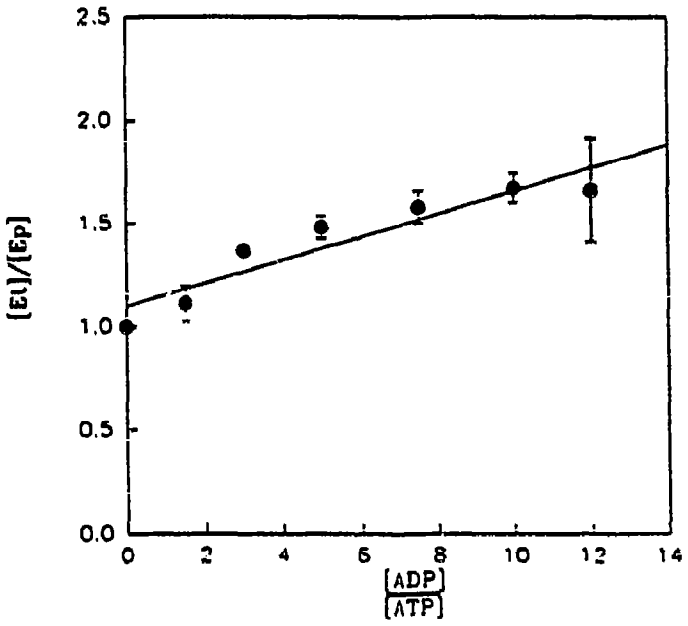

Fig. 3. Graphical representation of the K_{eq} determination. An aliquot of p56^{lyn} was autophosphorylated in the presence of 50 mM Tris-HCl pH 7, 25 mM $MgCl_2$, and 100 μM ATP at 30°C for 30 min. After 30 min, an equal volume of ADP was added to each sample to give final concentrations of the appropriate ratio relative to ATP. The samples were allowed to incubate a further 30 min and then analyzed by SDS-PAGE. The radioactive bands were cut from the gel and counted by liquid scintillation spectroscopy. The error bars represent standard deviation (n=3) and the line was drawn using linear regression.

the resolution of [^{3}H]ADP from [^{3}H]ATP obtained in a typical experiment by HPLC using a Waters 5 μ C_{18} column and an in-line Flow-Beta radioactivity flow detector to analyze the relative proportion of each nucleotide. The nucleotides were well resolved, with [^{3}H]ADP eluting at approximately 6 min and [^{3}H]ATP eluting at 8 min. In addition to the highly reproducible separation of the adenosine nucleotides, the ability to run the HPLC column isocratically enabled rapid analysis of the nucleotide composition of the reaction. Fig. 2B shows the time dependent formation of [^{3}H]ATP from [^{3}H]ADP. The reaction proceeded rapidly and plateaued by approximately 30 min with 65% of the total [^{3}H]ADP being converted to [^{3}H]ATP. The reaction did not proceed in the absence of added ATP (Fig. 2C) or Mg^{2+} (Fig. 2D), conditions essential for kinase autophosphorylation.

3.2. *Thermodynamic characterization of the p56^{lyn} autophosphorylation reaction*

The equilibrium constant (K_{eq}) of the autophosphorylation reaction:

$K_{eq} = \frac{[E_p][ADP]}{[E][ATP]}$, can be transformed into Equation (2):

$[E_t]/[E_p] + K_{eq}^{-1}([ADP/ATP]) + 1$ (2) where E_t represents total enzyme. To determine the K_{eq} the extent of autophosphorylation of p56^{lyn} at various ratios of ADP to ATP were measured and the results plotted according to Equation (2) (Fig. 3). From the slope of the line, the K_{eq} was calculated to be 3.31. The value of K_{eq} was also determined using longer incubation periods and similar values were obtained. The standard free energy of the reaction calculated from the K_{eq} was $\Delta G^{\circ}_{obs} = -0.72$ kcal/mol. Using the value for the free energy of hydrolysis of the β-γ bond of ATP to yield ADP and phosphate of −8.8 kcal/mol at pH=7 and [$MgCl_2$]=25 mM [9], the standard free energy of hydrolysis of phosphotyrosine-p56^{lyn} was determined to be −8.8 kcal/mol.

3.3. *Nucleoside diphosphate kinase activity of p56^{lyn}*

Previous investigators have shown that GTP can be used by pp60$^{c\text{-}src}$ as an alternate substrate to ATP [2]. If GDP can also be used as the substrate in the reversible reaction of p56^{lyn}, the enzyme would be expected to display nucleoside diphosphate kinase activity. A nucleoside diphosphate kinase reaction utilizing GDP and ATP as reactants (ATP + GDP ⇌ GTP + ADP) may be considered as the sum of the ATP mediated autophosphorylation and GDP induced reversal of the autophosphorylation reaction of the kinase.

Fig. 4 illustrates the time dependent formation of [^{3}H]GTP from [^{3}H]GDP and ATP. As observed with

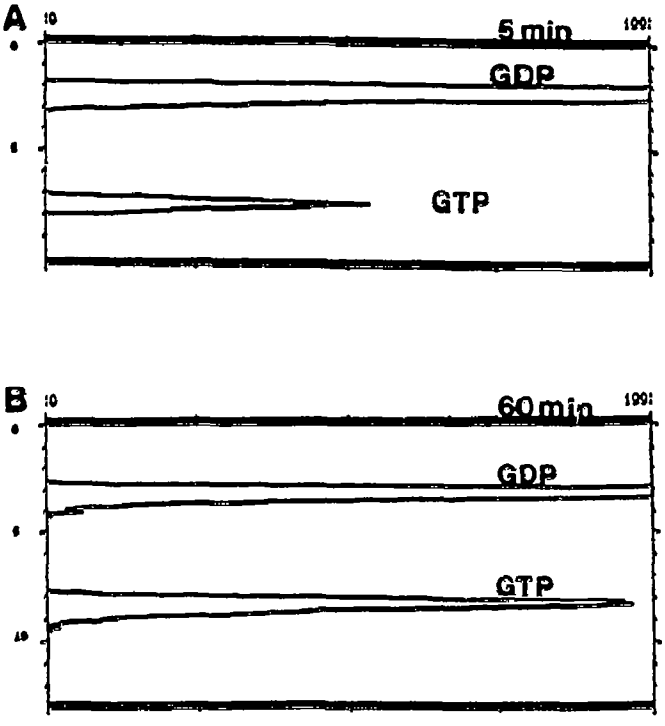

Fig. 4. p56^{lyn} catalyzed conversion of [^{3}H]GDP to [^{3}H]GTP. (A) Conversion of [^{3}H]GDP to [^{3}H]GTP during 5 min incubation. (B) Formation of [^{3}H]GTP catalyzed by p56^{lyn} during a 60 min incubation. An aliquot of p56^{lyn} was incubated in the presence of 50 mM Tris-HCl pH 7, 25 mM $MgCl_2$, 100 μM ATP, and 200 nM [^{3}H]GDP at 30°C. At the indicated times, the reaction was stopped by the addition to a final concentration of 5 mM GDP, 5 mM GTP and 50 mM EDTA. The reaction was analyzed by HPLC as outlined in section 2.

[^{3}H]ADP, the reaction did not proceed in the absence of Mg^{2+} or $p56^{lyn}$ (data not shown). Although detailed kinetic analysis of the $p56^{lyn}$ catalyzed GDP phosphorylation reaction has not been carried out, the observation that more than 15% of the GDP was converted to GTP within 5 min at an initial GDP concentration of 0.2 μM (Fig. 4A) suggests that the enzyme binds to GDP with relatively high affinity and catalyzes GDP phosphorylation with high efficiency.

In conclusion, $p56^{lyn}$, like a number of other protein tyrosine kinases, catalyses a reversible autophosphorylation reaction. Our analysis of this reaction has established a methodology which will enable further investigation of the reaction. In addition, the reversible autophosphorylation of $p56^{lyn}$ has been shown to be coupled with the phosphorylation of GDP to GTP in an ATP dependent manner under certain circumstances. Although $p56^{lyn}$ and other tyrosine kinases have been shown to catalyze a readily reversible phosphorylation reaction, the significance of this remains unknown. The apparent ratio of [ADP] to [ATP] was observed to be a limiting condition for $p56^{lyn}$ autophosphorylation. Because of the demonstrated role that kinase autophosphorylation has in the regulation of $pp60^{c\text{-}src}$ family kinase activity [7], the [ADP]/[ATP] ratio could serve as a secondary mechanism to modulate kinase activity in the enzyme microenvironment by decreasing the percent of autophosphorylated kinase. Alternately, this sensitivity could directly couple the relative kinase activity to the relative metabolic activity within the kinase microenvironment. While the concentration of ATP has generally been shown to exceed that of ADP in most tissues yet examined, certain tissues, such as bovine mesenteric artery, have been determined to contain equivalent or greater concentrations of ADP relative to ATP [10]. Similary, while the significance of the ability of $p56^{lyn}$ to catalyze the ATP dependent phosphorylation of GDP to GTP has not been established, it remains a formal possibility that this reaction may be of physiological relevance under certain conditions within the kinase microenvironment in vivo.

Acknowledgements: We thank Dr. H. Zwiers, University of Calgary, for allowing us to use his in line radioactivity detector. We also appreciate the technical assistance of Ms. Faith Hwang. C.M.E.L. is the recipient of an Alberta Heritage Foundation for Medical Research Studentship. J.H.W. is an Alberta Heritage Foundation for Medical Research Scientist. This work was supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Alberta Heritage Foundation for Medical Research.

REFERENCES

[1] Fukami, Y. and Lipmann, F. (1983) Proc. Natl. Acad. Sci. USA 80, 1872–1876.
[2] Feder, D. and Bishop, J.M. (1990) J. Biol. Chem. 265, 8205–8211.
[3] Foulkes, J.G., Chow, M., Gorka, C., Frackelton, A.R. and Baltimore, D. (1985) J. Biol. Chem. 260, 8070–8077.
[4] Hubler, L., Gill, G.N. and Bertics, P.J. (1989) J. Biol. Chem. 264, 1558–1564.
[5] Shizuta, Y., Beavo, J.A., Bechtel, P.J., Hoffmann, F. and Krebs, E.G. (1972) J. Biol. Chem. 250, 6891–6896.
[6] Litwin, C.M.E., Cheng, H.C. and Wang, J.H. (1991) J. Biol. Chem. 266, 2557–2566.
[7] Cooper, J.A., in: Peptide and Protein Phosphorylation (B. Kemp, Ed.) Uniscience CRC Press, Boca Raton, FA, 1990, pp. 85–111.
[8] Laemmli, U.K. (1970) Nature 227, 680–689.
[9] Alberty, R.A. (1969) J. Biol. Chem. 244, 3290–3302.
[10] Butler, T.M. and Davies, R.E., in: Handbook of Physiology, Section 2: Cardiovascular Smooth Muscle Vol. II (D.F. Bohr, A.P. Somlyo and H.V. Sparks, Eds.; S.R. Geiger, Exec. Ed.), Waverley Press, Baltimore, 1980, pp. 237–252.