Volume 292, number 1,2, 69–72 FEBS 10318 November 1991

ADONIS 001457939101028P

Spectrophotometric determination of functional characteristics of protein kinases with coupled enzymatic assay

Zusana Technikova-Dobrova[3], Anna Maria Sardanelli[2] and Sergio Papa[1]

[1]*Institute of Medical Biochemistry and Chemistry and* [2]*Centre for the Study of Mitochondrial and Energy Metabolism, CNR, University of Bari, Bari, Italy* and [3]*Institute of Microbiology, Czechoslovak Academy of Science, Praha, Czechoslovakia*

Received 30 August 1991

The performance of enzyme-coupled spectrophotometric assay of protein kinases and its merits as compared with the radioisotopic method in characterizing functional activity of protein kinases are described. Kinetic parameters of protein kinase C and protein kinase A so obtained are presented. General application of the spectrophotometric assay in the study of protein kinases is recommended.

Protein kinase; Protein kinase C; Protein kinase A; Protein kinase assay

1. INTRODUCTION

Recognition of the reversible phosphorylation of proteins as a major regulatory mechanism in eukaryotic [1,2] and prokaryotic cells [3] has given rise to intensive research on protein kinases and phosphatases which have become a topical issue in cellular biochemistry and pathology.

The various protein kinases, described in eukaryotes [1,4,5] and prokaryotes [6], catalyze the transfer of the γ-phosphoryl group of ATP or GTP to specific amino acid residues in protein substrates. Protein kinases and their substrates, only some of which have been so far identified, have been detected following the incorporation of radioactive γ-phosphoryl group from [^{32}P]nucleoside triphosphate into an appropriate substrate. This radioisotopic assay is generally used to characterize the functional activity of kinases [7–12].

This paper describes the performance of an enzyme-coupled spectrophotometric assay of protein kinases and examines its merits, as compared with the radioisotopic assay, in characterizing functional activity of protein kinases. A spectrophotometric assay of protein kinase was used by Cook et al. in 1982 [13], see also [14], but to our knowledge, since then, it has been applied only by the same group to a kinetic study of protein kinase A [15]. It is proposed here that a general application of the spectrophotometric assay would facilitate the study of protein kinases and their role in protein phosphorylation.

LDH, lactate dehydrogenase; PK, pyruvate kinase; PKC, protein kinase C; PKA, cAMP dependent protein kinase A catalytic subunit; PEP, phosphoenolpyruvate

Correspondence address: S. Papa, Institute of Medical Biochemistry and Chemistry, University of Bari, Piazza Giulio Cesare, 70124 Bari, Italy. Fax: (39) (80) 278429.

2. MATERIALS AND METHODS

2.1. *Materials*

DE-52, AH Sepharose 4B and phenyl-Sepharose were purchased from Pharmacia. Lactate dehydrogenase (LDH; EC 1.1.1.27), pyruvate kinase (PK; EC 2.7.1.40), phosphoenolpyruvate, NADH and ATP were from Boehringer-Mannheim. Protamine sulfate (salmine; mol. wt. 6000–7000) and HEPES buffer were from Sigma. Staurosporine, Streptomyces sp was from Calbiochem. [γ-^{32}P]ATP, 3000 Ci/mmol was from Amersham International.

2.2. *Protein kinase preparations*

Protein kinase C (PKC) was purified from rat brain by sequential DE-52/threonine-Sepharose/phenyl Sepharose chromatography as in [7]. A sample of PKC purified from bovine brain, with a specific activity of 400 nmol·min^{-1}·mg prot^{-1}, measured with the radioisotopic assay [16], was kindly provided by Prof. A. Azzi, Bern. The catalytic subunit of cAMP-dependent protein kinase (PKA) purified from bovine heart [17], with a specific activity of 1400 nmol·min^{-1}·mg prot^{-1}, measured with the radioisotopic assay, was kindly provided by Dr. P. Tortora, Milan.

2.3. *Spectrophotometric assay of protein kinase activity*

Reaction 1, catalyzed by protein kinase, is coupled in the presence of added PK, PEP, LDH and NADH to reactions 2 and 3. NADH oxidation (reaction 3), measured directly and continuously in the incubation mixture, provides an immediate estimate of the rate of reaction 1, with the concentrations of the reagents set up so that reaction 1 is the rate-limiting step.

$$\text{ATP + protein substrate} \xrightarrow{\text{Protein kinase}} \text{ADP + phospho-protein} \quad (1)$$

$$\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + pyruvate} \quad (2)$$

$$\text{Pyruvate + NADH} \xrightarrow{\text{LDH}} \text{lactate + NAD}^{+} \quad (3)$$

The reaction mixture contained: (A) Tris-HCl 20 mM, pH 7.4 and

$MgCl_2$ 5 mM for PKC or (B) K^+-phosphate buffer 20 mM, pH 7.4 and 5 mM $MgCl_2$ for PKA. To 1.5 ml of the reaction mixture in the spectrophotometric cuvette, under rapid stirring and thermostatically controlled at 25°C, NADH, PK and LDH, PEP, the kinase sample, ATP and protamine sulphate were added at the concentrations given in the figure legends. NADH oxidation was monitored with a conventional spectrophotometer (Perkin Elmer, lambda 5) at a signal output of 0.1 OD full scale (20 cm) at 340 nm, $\Delta\varepsilon$ reduced-oxidized = 6.22 $mM^{-1}\cdot cm^{-1}$, with a sensitivity of 250 nM at a signal/noise ratio of 10, or with a dual wavelength spectrophotometer (Johnson Foundation, Philadelphia) at 340–374 nm $\Delta\varepsilon$ = 6.0, or 360-374 nm $\Delta\varepsilon$ = 2.0, with possible output amplification up to 8×10^{-3} OD full scale and corresponding sensitivities of 25 and 75 nM at a signal/noise ratio of 10 with an instrumental drift lower than 1×10^{-4} Δ OD/min.

2.4. *Protein determination*

Protein concentrations were determined by a modified Lowry-Folin assay [18].

3. RESULTS

Fig. 1 shows the spectrophotometric determination of protein kinase activity in crude extract of rat brain which has a relatively high content of PKC [4,19]. The assay was carried out in the dual-wavelength spectrophotometer at 360–374 nm to minimize interference from turbidity changes. The spectrophotometric trace shows that the brain extract contained traces of NADH oxidase activity. The addition of ATP elicited an ATPase activity, resulting in the oxidation of NADH from the sum of reactions 4, 2 and 3.

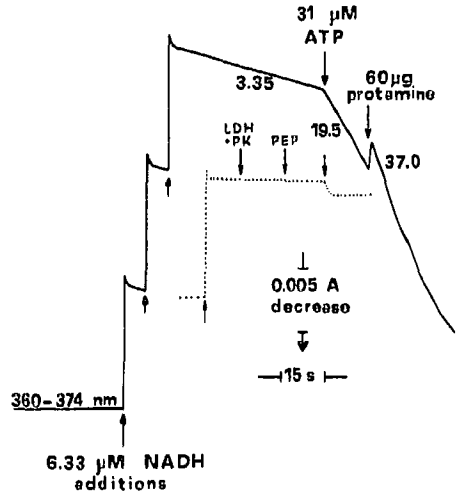

Fig. 1. Determination of protein kinase activity in crude extract of rat brain. 760 μg protein of the supernatant of rat brain homogenate, obtained after 2 h centrifugation at 105000 × g, was suspended per ml of reaction mixture (A), containing 3.1 units/ml ($\mu M \cdot min^{-1}$) each of LDH and PK and 235 μM PEP. 6.33 μM NADH additions were made as indicated and served as internal sensitivity standards. The other additions are specified on the illustration. The figures on the traces refer to rates of NADH oxidation in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. The dotted line shows a control in the absence of the brain extract. The small deflection caused by ATP addition is due to the trace of contaminating ADP (0.2%). For other details see under Materials and Methods and text.

$$ATP + H_2O \xrightarrow{\text{ATPase}} ADP + Pi \quad (4)$$

The addition of protamine, which functions as a general substrate of protein kinases, also in the absence of the specific activators required with other protein substrates [4], resulted in an increase in the rate of NADH oxidation, which, after correction for that associated with NADH oxidase and ATPase activities, gave a specific activity for protein kinase of 17 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Fig. 2 illustrates the spectrophotometric determination of the activity of PKC purified from rat brain. The assay showed very low overall NADH oxidase and ATPase activity. The addition of 15 μg protamine resulted in an immediate rapid oxidation of NADH, giving, after correction for unrelated NADH oxidation, a specific activity for PKC of 223. This continued until all protamine had been phosphorylated and NADH oxidation returned to the rate measured before its addition. It can be noted that the addition of 15 μg protamine, giving a concentration of 1.37 μM (mol. wt. 6000–7000) resulted in the utilization of 4.25 μM ATP. This shows that ca. 3 residues per mol of protamine are phosphorylated. An excess of protamine elicited a maximal rate of NADH oxidation giving a specific activity for PKC of 385. NADH oxidation caused by the addition of protamine was completely blocked by staurosporine, a potent inhibitor of PKC [20].

The spectrophotometric assay gave a linear Lineweaver-Burk plot for saturation kinetics of rat brain PKC with respect to ATP (Fig. 3), from which a V_{max}

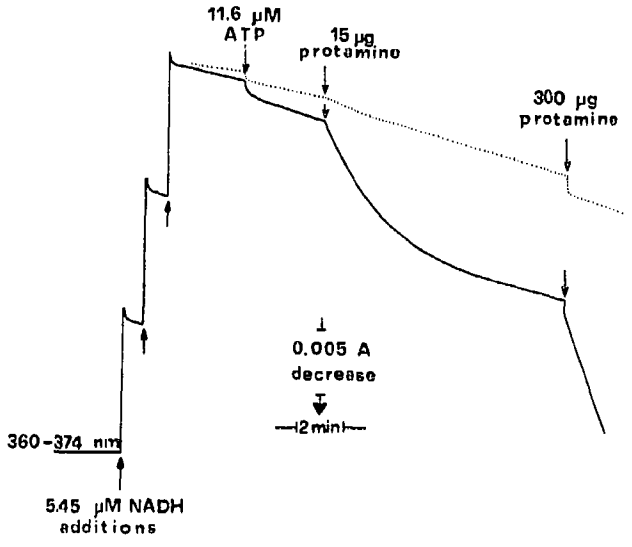

Fig. 2. Determination of rat brain PKC activity. 7.5 μg protein of purified rat brain PKC (eluate of phenyl-Sepharose chromatography [7]) was suspended per ml of reaction mixture (A), containing LDH, PK and PEP as specified under Materials and Methods and in the legend to Fig. 1. All the other additions are indicated in the illustration. The dotted trace refers to an experiment carried out under exactly the same conditions of the measurement of the solid trace but in the presence of 85 μM staurosporine.

of 650 and a K_m for ATP of 14 μM can be calculated. A saturation experiment of rat brain PKC with protamine gave for this substrate a K_m of 1.8 μM. Similar kinetic patterns were obtained with the sample of bovine brain PKC.

Spectrophotometric assay of the activity of PKA showed this to be linear with protein concentration in the range from 70 to 9000 ng of enzyme protein. Spectrophotometric analysis of saturation kinetics of PKA with ATP (in the presence of 27 μM protamine) and with protamine (in the presence of 150 μM ATP) gave, in both cases, straight lines by Lineweaver-Burk plots, from which a V_{max} of 1500 nmol·min^{-1} mg protein^{-1} and K_m for ATP of 6 μM and K_m for protamine of 2.8 μM, respectively, were obtained.

Fig. 4 shows spectrophotometric analysis of the pH dependence of PKC and PKA activities.

4. DISCUSSION

The results presented show the spectrophotometric enzyme-coupled assay to suitable for easy, rapid and sensitive analysis of functional activity of protein kinases. Compared to the radioisotopic assay the spectrophotometric method has the obvious advantage of avoiding costly [^{32}P]nucleotides and safety restrictions while the same or smaller amounts of proteins can be conveniently assayed. The sensitivity of the spectrophotometric assay is ca. 250 nM with conventional spectrophotometers and ca. 30 nM with dual-wavelength spectrophotometers. The latter instrument is particularly suitable to avoid light-scattering and turbidity artifacts which could arise in crude extracts or when phospholipid suspensions [21] or micelles [22] have to be used to activate PKC. The spectrophotometric assay also allows (in the same sample) contaminating ATPase activity to be measured and corrected for. The ATPase activity can, on the other hand, represent a serious complication for the radioisotopic assay.

The spectrophotometric assay, providing a direct and continuous recording of the enzymatic activity appears, is the method of choice for the kinetic study of protein kinases as well as for the identification and functional analysis of their inhibitors and activators.

Another advantage of the spectrophotometric assay derives from the fact that the concentration of ATP is maintained at a constant level in the course of the reaction by continuous regeneration by pyruvate kinase.

The spectrophotometric assay can be used with any natural and synthetic polypeptide substrate of protein kinases. Also in this respect it has an advantage over the radioisotopic assay, which requires separation of [^{32}P]protein substrates from [γ-^{32}P]nucleotides, a process which needs rigorous procedures and is difficult to achieve with certain polypeptide substrates [13,14].

Some protein kinases also display protein phosphatase activity or can be contaminated by phosphatases [1]. Both will result in the hydrolytic removal of phosphate from the phosphorylated protein substrate and underestimate or prevent measurement of the kinase activity with the radioisotopic assay. The phosphatase activity will not prevent the spectrophotometric assay of the kinase activity. Rather the phosphatase reaction, being coupled with reactions 1, 2 and 3 (see section 2) can also be detected in the spectrophotometric assay. Finally the spectrophotometric assay can provide an estimate of the purity and/or the molecular weight of protein substrates yet to be characterized, or of the number of phosphoryl groups incorporated per mol of substrate protein.

Obviously the radioisotopic assay remains essential for rapid screening of tissues and cells to identify pro-

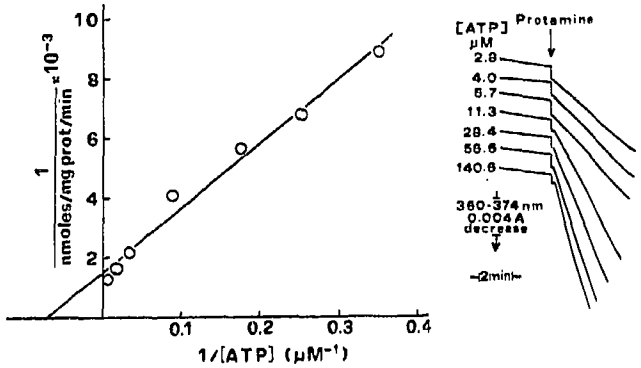

Fig. 3. Spectrophotometric analysis of saturation kinetics of rat brain PKC. 5 μg protein of purified rat brain PKC was incubated per ml of reaction mixture (A) containing LDH, PK and PEP as specified in the legend to Figs. 1 and 2. The concentration of ATP was varied in different samples as indicated in the illustration. The kinase reaction was started by the addition of 180 μg protamine/ml (27.7 μM). For experimental conditions and other details see under Materials and Methods, legend to Fig. 2 and text.

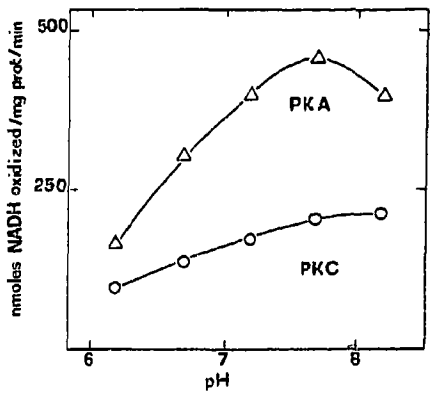

Fig. 4. Spectrophotometric analysis of pH-dependence of activity of rat brain PKC and bovine heart PKA. 7.8 μg protein of purified rat brain PKC or 7.3 μg protein purified PKA was suspended per ml of a mixture containing: 20 mM Tris-HCl; 20 mM HEPES; and 5 mM $MgCl_2$. The mixtures were adjusted to the pHs indicated in the illustration, and the reagents are as specified in the legend to Fig. 2. ATP=145 μM. The reaction was started with the addition of 34 μg/ml of protamine.

tein kinase enzymes and/or isoenzymes or their genetically-engineered forms and variants and their protein substrates.

Acknowledgements: This work was in part supported by Grant 89.00317.75 of C.N.R., Italy. Dr. Z. Technikova-Dobrova was the recipient of a travel grant from the University of Bari. The authors thank Prof. J.M. Tager for reading the manuscript and for his suggestions.

REFERENCES

[1] Boyer, P.D. and Krebs, E.G. (eds.) (1986) in: Control by Phosphorylation, part A. The Enzymes, vol. XVII, Academic Press, New York.
[2] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.
[3] Cozzone, A.J. (1988) Annu. Rev. Biochem. 42, 97–125.
[4] Kikkava, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–44.
[5] Taylor, S.S., Buechler, J.A. and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005.
[6] Saier, Jr. M.H., Wu, L.F. and Reizer, J. (1990) Trends Biochem. Sci. 15, 391–395.
[7] Kitano, T., Masayoshi, G., Kikkava, U. and Nishizuka, Y. (1986) Methods Enzymol. 124, 349–352.
[8] Yasuda, I., Kishimoto, A., Tanoka, S., Tominaga, M., Sakurai, A. and Nishizuka, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1220–1227.
[9] Wylie, D., Stock, A., Wong, C.Y. and Stock, J. (1988) Biochem. Biophys. Res. Commun. 151, 891–896.
[10] Banerjee, C. and Sarkar, D. (1990) J. Gen. Microbiol. 136, 1051–1057.
[11] Bhatnagar, D., Glass, D.B., Roskoski, Jr. R., Lessor, R.A. and Leonard, N.J. (1988) Biochemistry 27, 1988–1994.
[12] Roskoski, Jr. R., and Ritchie, P. (1991) J. Neurochem. 56, 1019–1023.
[13] Cook, P.F., Neville, M.E., Vrana, K.E., Hartl, F.T. and Roskoski, Jr. R., (1982) Biochemistry 21, 5794–5799.
[14] Roskoski, Jr. R. (1983) Methods Enzymol. 99 (part F), 3.6.
[15] Yoon, M.-Y. and Cook, P.F. (1987) Biochemistry 26, 4118–4125.
[16] Mahoney, C.W. and Azzi, A. (1988) Biochem. Biophys. Res. Commun. 154, 694–697.
[17] Demaille, J., Peters, K.A. and Fisher, E.H. (1977) Biochemistry 16, 3020–3086.
[18] Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem 85, 295–300.
[19] Kikkava, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348.
[20] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 91, 1218–1224.
[21] Nelsestuen, G.L. and Bazzi, M.D. (1991) J. Bioenerg. Biomembr. 23, 43–61.
[22] Bell, R.N., Hannun, Y. and Loomis, C. (1986) Methods Enzymol. 124, 353–359.