Volume 331, number 1,2, 114–118 FEBS 13018 September 1993
© 1993 Federation of European Biochemical Societies 00145793/93/$6 00

Bovine inositol monophosphatase

Studies on the binding interactions with magnesium, lithium and phosphate ions

P.J. Greasley, M.G. Gore*

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, S09 3TU, UK

Received 3 August 1993

Rapid equilibrium dialysis has been used to show that recombinant bovine brain inositol monophosphatase binds one equivalent of P_i per subunit of enzyme. P_i is only bound in the presence of Mg^{2+} ions. The dissociation constant for the equilibrium is approximately 50 μM. This value of K_d is independent of the concentration of the Mg^{2+} ions and of the presence or absence of Li^+ ions. Lithium ions which inhibit the enzyme uncompetitively are not able to support the binding of the P_i to the enzyme The observation that P_i only binds in the presence of Mg^{2+} ions supports similar conclusions made in experiments which studied the protection of the enzyme from proteolytic degradation and chemical modification.

Inositol monophosphatase; Magnesium; Lithium; Phosphate; Rapid equilibrium dialysis

1. INTRODUCTION

Inositol monophosphatase is a dimeric enzyme of subunit molecular weight 30,055 Da which catalyses the final dephosphorylation of inositol 1, 3 and 4 monophosphate [1]. The enzyme has an absolute requirement for Mg^{2+} ions for catalytic activity [2], although higher concentrations of this ion leads to inhibition of the enzyme (uncompetitive relative to substrate) [2]. The enzyme is also inhibited in an uncompetitive manner by Li^+ ions which is thought to account for the therapeutic value of this metal ion in the treatment of some manic disorders [3]. The K_m and K_i values for these metals is strongly dependent upon the nature of the substrate [4]. The structure of the recombinant human brain enzyme has now been determined at 2.1 Å resolution by X-ray crystallography [5] and has allowed detailed examination of the amino acid residues implicated in ligating the metal ions [6,7]. Recently the kinetic mechanism of the enzyme has been studied [8]. It has been proposed that the enzyme can bind inositol 1-phosphate or Mg^{2+} in a random order process and that the inositol moiety is the first product to leave the enzyme after the hydrolysis has taken place. The proposed mechanism then suggests that the Mg^{2+} ion leaves the $E{\cdot}Mg{\cdot}P_i$ complex followed by the phosphate ion. The proposed reaction scheme is summarised in Fig. 1. The inhibitory interaction of Li^+ ions occurs with either the $E{\cdot}Ins{\cdot}P_i$ or the $E{\cdot}P_i$ complexes upon dissociation of the catalyctic Mg^{2+} ion from the enzyme product complexes [8]. Since the inhibitory effects of Mg^{2+} and Li^+ have been shown to be mutually exclusive [9], they probably bind at the same site or at two overlapping sites within the enzyme. It has been proposed that the inhibitory site and the site which binds the catalytically important Mg^{2+} ion need not be the same [9]. Indeed, at high concentrations Li^+ behaves as a non-competitive inhibitor with respect to substrate and is believed to interact at the same site as the catalytic Mg^{2+} ion [9]. Although the most simple description for the interaction of this enzyme with the metals might support the concept of a single metal binding site in which the different interactions depend upon the absence or presence of substrate, a model proposing two metal binding sites per subunit of enzyme cannot be excluded. It is apparent from the proposed reaction scheme (see Fig. 1) that the compulsory order of dissociation of products from the complexes requires that the binding of the P_i in the reverse direction can occur in the absence of Mg^{2+} ions.

Several studies on the structure of the enzyme and chemical modification of reactive amino acid residues, e.g. protection from proteolysis [10], prevention of quenching of protein fluorescence whilst undergoing modification by the thiol reagent, *N*-ethylmaleimide [11], and protection from loss of activity in the presence of the arginine-specific reagent, phenylglyoxal [12], showed that protection by P_i could only take place if Mg^{2+} ions were also present.

Recent work [13] has highlighted the similarity between inositol monophosphatase and fructose 1,6-bisphosphatase (which has a requirement for Mg^{2+} ions for activity) in terms of a conserved $\alpha\beta\alpha\beta\alpha$ tertiary structure and in particular a conserved motif including an

*Corresponding author.

 Published by Elsevier Science Publishers B.V

array of acid residues known to be involved in metal ligation in the fructose 1,6-bisphosphatase [14]. This conserved site has been suggested to be the putative Mg^{2+} binding site in inositol monophosphatase [5]. Fructose 1,6-bisphosphatase also shares other features with the inositol monophosphatase, for example, it too is inhibited by Li^+ ions, contains a highly susceptible proteolytic site (K36–S37 in inositol monophosphatase [10,15]; T66–G67 in fructose 1,6-bisphosphatase [16]), and like the inositol monophosphatase undergoes a structural change during the catalytic reaction [17]. In this enzyme the binding of phosphate requires the presence of Mg^{2+} ions [18].

In this paper we describe the results of rapid equilibrium dialysis experiments which were used to explore the interactions between the inositol monophosphatase, Mg^{2+} ions, Li^+ ions and P_i ions and to determine whether Mg^{2+} ions are required for P_i binding.

2. MATERIALS AND METHODS

2.1. *Enzyme*

The growth of bacterial cultures and the isolation and purification of the enzyme has been described elsewhere [19]. All laboratory reagents were purchased from Sigma Chemical Co. Poole, UK, or Merck Ltd., Poole, UK.

^{32}P-Labelled P_i was purchased from Amersham, UK and was carrier free.

2.2. *Enzyme activity*

Activity was measured by the continuous fluorimetric method described by Gore et al. [20].

2.3. *Protein concentration*

The concentration of the enzyme was determined by use of the bichinconinic acid method as described by Smith et al. [21].

2.4. *Buffers*

The enzyme was dissolved in 50 mM Tris-HCl buffer, pH 8.0, at 21°C and this buffer was also used for dialysis and for rapid equilibrium dialysis experiments. Metal ions were removed by initial dialysis against the same buffer containing 1 mM 1,10 phenanthroline, 1 mM EGTA and 1 mM EDTA. The chelating agents were then removed by subsequent dialysis against buffer alone. All solutions were prepared in Analar grade water and glassware was acid washed and rinsed in Analar water

2.5. *Rapid equilibrium dialysis*

A small apparatus was constructed consisting of a cyllinder of perspex (2 × 1 cm diameter) on to one end of which was placed a dialysis membrane. The cylinder was mounted onto a block of perspex through which a continuous stream of buffer (12 ml per min) was passed and collected in a fraction collector. The method has been fully described elsewhere [22].

2.6. *Scintillation counting*

All samples were counted on a Phillips PW4700 liquid scintillation counter using Optiphase, Hisafe III (Lablogic, Sheffield, UK).

3. RESULTS

500 μl of Tris-HCl buffer (pH 8.0) containing 25 μM of P_i seeded with 1 μl of $^{32}P_i$ was placed and stirred in the upper compartment of the device and the amount of radioactive P_i traversing the membrane was monitored as increasing amounts of enzyme were added to the solution (Fig. 2, also see Fig. 4). This experiment permitted us to determine whether all of the radiolabel could be eventually bound to the enzyme, and allowed an estimation of K_d. Alternatively, 1 μl of the carrier-free radioactive $^{32}P_i$ (10 μCi) was added to a stirred solution of 1 ml of the enzyme (133 μM) in the reaction chamber above the dialysis membrane and that radioactivity which passed into the stream of buffer in the lower block was collected and quantified by liquid scintillation counting in the presence of a water-miscible scintillint. The concentration of P_i in the enzyme solution was increased by addition of non-radioactive P_i. The dialysate was collected for 2 min after each addition (4 × 6 ml fractions) and the increased flux of radioactive P_i coming through the membrane was determined (Fig. 3, also see Fig. 4).

The four fractions from each addition were counted individually and the last three of each batch were averaged. The individual points plotted are the average of the last three fractions collected after each addition and these typically had standard deviations of less than 3% (the first fraction was ignored since the system required approximately 15 s to reach equilibrium). In all of the experiments the total loss of radioactivity from the test incubation to the buffer stream was less than 0.75%. In order to check that none of the added radioactive $^{32}P_i$ was adsorbed onto the surfaces of the apparatus the experiment was repeated. This time the enzyme was added to the apparatus in the presence of a starting concentration of 25 μM non-radioactive P_i to which was added the small sample of carrier-free $^{32}P_i$. This control showed that the amount of radiolabel passing through the membrane was the same as when no non-radioactive P_i was present, and therefore that none of the initial 'seed' of radiolabelled $^{32}P_i$ was lost from solution due to adsorption to the apparatus.

Fig. 2 shows the decrease in flux of $^{32}P_i$ across the membrane as the concentration of enzyme was increased. Analysis of the curve (Fig. 4) showed that all of the radioactivity could be bound by infinitely high concentrations of the enzyme, i.e. theoretically, a situation could be reached where no radioactivity passed across the membrane. Examination of the binding curve (shown in Fig. 2, inset) showed that the K_d for the equilibrium between P_i and the enzyme (in the presence of 4 mM Mg^{2+} ions) under these conditions is 50 μM and that approximately 1 equivalent of P_i binds per subunit of enzyme. Fig. 3 displays data obtained from experiments where the amount of $^{32}P_i$ displaced from a binary complex with the enzyme was monitored as the concentration of non-radioactive P_i was increased. For the experiment performed under the same conditions as that described in Fig. 2 the value of K_d obtained was 50 μM (see Figs. 3 and 4) supporting the previous data.

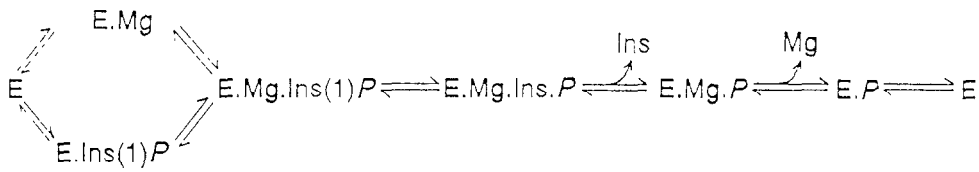

Fig. 1 The proposed mechanism of the reaction catalysed by inositol monophosphatase as described by Leech et al. [8]. The reaction is proposed to proceed via a quaternary complex of E·Ins(1)P·Mg^{2+}·H_2O. The enzyme may be inhibited uncompetitively by the presence of Li^+ ions or by high concentrations of Mg^{2+} ions after the hydrolysis of the substrate–phosphate ester but before phosphate is released.

When experiments were repeated at different concentrations of Mg^{2+} ions (200 μM–50 mM) the estimates of K_d and the stoichiometry of the binding reactions remained unchanged. However, if no Mg^{2+} ions were included then the level of $^{32}P_i$ crossing the membrane per fraction was independent of the concentration of P_i or enzyme and occurred at a level equivalent to totally free P_i. This observation confirms our previous deductions that Mg^{2+} has to be present for P_i to bind. The K_d for P_i from the enzyme·Mg^{2+}·P_i complex was found to be 50 ± 5 μM at Mg^{2+} concentrations between 200 μM and 50 mM.

The presence of Li^+ ions were found to be unable to support the binding of P_i (Fig. 3) the flux of radiolabel across the membrane being the same as that which occurs when the same amount of $^{32}P_i$ is placed alone in the upper compartment of the device. Furthermore it was found that the presence of 10 mM Li^+ ions have no effect on the K_d for P_i determined in the presence of 200 μM–50 mM Mg^{2+}.

4. DISCUSSION

The proposed mechanism of inositol monophosphatase is summarised in Fig. 1. The binding of the Mg^{2+} ion with respect to the substrate is random, but with respect to the second product and the competitive inhibitor P_i is ordered. This necessitates that P_i binds to the enzyme in the reverse direction in the absence of the Mg^{2+} ion. Fig. 3 shows the result of a rapid equilibrium dialysis experiment in which $^{32}P_i$ was incubated with enzyme and then an increasing concentration of unlabelled P_i was added. The transfer of $^{32}P_i$ across the membrane was not influenced by the increasing [P_i] and the number of dpm transferring across the membrane was the same as in the absence of the enzyme (the de-

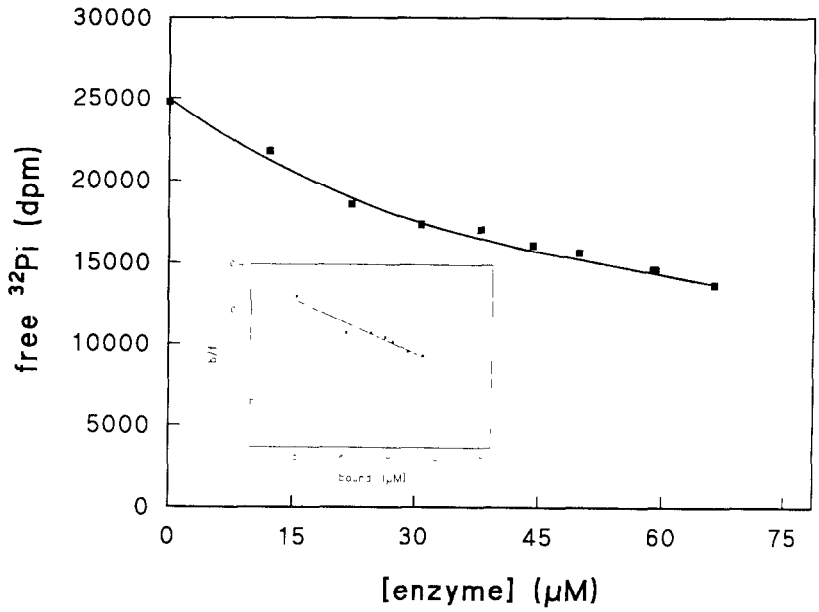

Fig. 2. The effect of increasing enzyme concentration on the level of free $^{32}P_i$. 1 μl of carrier-free $^{32}P_i$ (10 mCi/ml) was added to 500 μl of 50 mM Tris-HCl, pH 8.0, at 21°C also containing 25 μM P_i and 8 mM Mg^{2+} ions. The collection of fractions of the eluate was initiated and aliquots of an enzyme solution (also containing the same levels of P_i and Mg^{2+}ions as above) were sequentially added to the dialysis device. The concentration of P_i and Mg^{2+} ions remained unchanged but the concentration of enzyme increased. The figure shows the decrease in flux of free $^{32}P_i$ across the membrane after each addition of enzyme. The inset shows an analysis (bound/free against bound with respect to enzyme) of the binding curve.

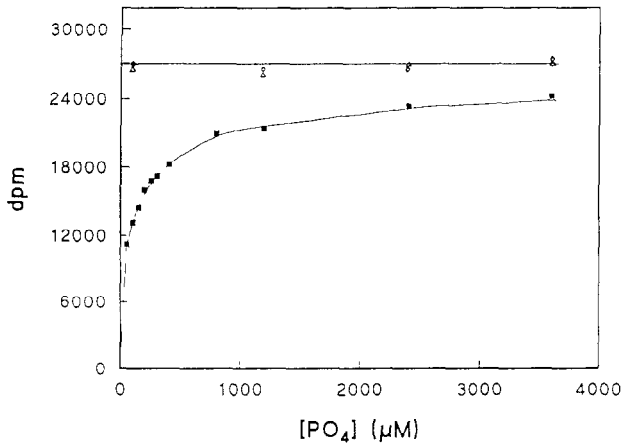

Fig. 3 The effect of increasing P_i on the flux of $^{32}P_i$ across the membrane. 1 μl of carrier-free $^{32}P_i$ was added to 1 ml of 133 μM enzyme in 50 mM Tris-HCl, pH 8.0, at 21°C also containing 8 mM Mg^{2+} ions. The fraction collection was initiated and the concentration of P_i in the upper chamber increased by sequential addition of aliquots of P_i present as K_2HPO_4 (■). The experiment was repeated under identical conditions except that the enzyme was omitted (○). Finally, an experiment was performed in the presence of enzyme but the Mg^{2+} ions were replaced by 10 mM Li^+ ions (△).

creasing specific radioactivity is compensated exactly by the increased rate of diffusion). Fig. 2 also shows that in the presence of Mg^{2+} the transfer of $^{32}P_i$ across the membrane is much lower, implying that more was being bound by the enzyme. The addition of increasing concentrations of non-radiolabelled P_i to the incubation led to the displacement of the $^{32}P_i$ and enabled the determination of a K_d of 50 μM for P_i from the enzyme·Mg^{2+} complex. In an analogous experiment, rather than titrating P_i and monitoring the displacement of $^{32}P_i$, inositol monophosphatase was titrated into 500 μl of 50 mM Tris-HCl (pH 8.0) containing 25 μM P_i (inc. 10 μCi $^{32}P_i$). As the concentration of enzyme was increased the level of free P_i was decreased. Analysis of these two experiments is summarised in Fig. 4. It is apparent that the binding phenomenon is dependent upon the concentration of enzyme and the concentration of P_i. However, the interaction in both cases is Mg^{2+} dependent. This observation is in direct contrast to that necessitated by the mechanism in Fig. 1. In a mechanism in which there is a single metal binding site which has both catalytic and inhibitory function it is necessary that the catalytic metal ion dissociates to allow the binding of the inhibitory ion, as described in Fig. 1. The dependence therefore of P_i binding upon the presence of Mg^{2+} suggests that there may be two sites within inositol monophosphatase. The inhibition of inositol monophosphatase by the mutually exclusive metal ions traps a phosphate/enzyme intermediate [9]. If the Mg^{2+}-dependent P_i binding detected by rapid equilibrium dialysis is the inhibited P_i·Mg^{2+}·E complex then Li^+ should also support the formation of the analogous P_i·Li^+·E complex if the two are truely mutually exclusive. Fig. 3 shows that Li^+ does not support the binding of P_i to the enzyme suggesting that the metal binding site which supports the binding of the P_i is not that which binds the inhibitory metal ions. The K_d value obtained for P_i (50 μM) is approximately 10-fold lower than that determined previously for the K_i for P_i [8]. In kinetic experiments conducted in parallel with the rapid equilibrium dialysis utilising the same experimental conditions, the K_i was determined to be 60 μM. Other workers have shown the K_i for P_i to be dependent upon the concentration of Mg^{2+} [8] and we have made similar observations. In contrast though, the K_d, as determined by the method of rapid equilibrium dialysis, showed no such concentration dependence. It has been previously reported that increasing the concentration of P_i leads to a decrease in the K_i for Li^+ [8], and increasing the concentration of Li^+ or Mg^{2+} the affinity for P_i is also raised. We have found that in the rapid equilibrium experiments, the additional presence of Li^+ caused no enhancement in the affinity of the enzyme for P_i. Since Mg^{2+} and Li^+ exert similar effects on the K_i for P_i inhibition, it seems probable that this feature is due to metal occupation of a single inhibitory site. The failure to potentiate binding of P_i by Li^+ or higher concentrations of Mg^{2+} ions in these experiments implies that under these conditions only a single, non-inhibitory metal binding site is being occupied. This observation is more significant since the K_i for Mg^{2+} and Li^+ has been shown to be dependent upon the nature of the substrate [9]. This may imply that the inhibitory site for both metal ions may only exist in an enzyme intermediate complex and not in a ground state E·Mg^{2+}·P_i complex. The relationship of Mg^{2+} concen-

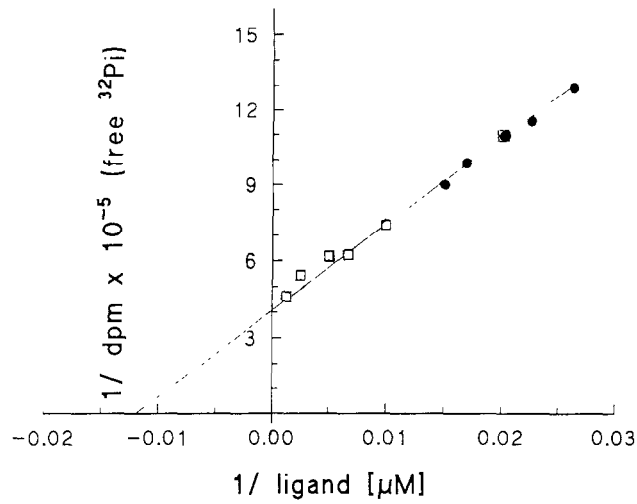

Fig 4. A double reciprocal analysis of data shown in Figs. 2 and 3. The figure shows the decrease or increase of $^{32}P_i$ flux when enzyme is added to 25 μM P_i (containing $^{32}P_i$) (●), or P_i is added to a solution of E·$^{32}P_i$ complex (□), respectively. The data from each experiment were normalised before plotting in order to compensate for radioactive decay of $^{32}P_i$.

tration on the activity of the enzyme has often been shown to demonstrate positive co-operative effects (Hill coefficient = 2) [9]. Whereas these might have implied subunit–subunit interactions (1 equivalent of Mg^{2+} per subunit) they may now be indicative of interactions between two metal ions on the same subunit.

The structural similarity and shared metal binding motif of inositol monophosphatase and fructose 1,6-bisphosphatase suggests that the interactions of metal ligands with these enzymes may be similar. It has recently been demonstrated that the metal binding motif in fructose 1,6-bisphosphatase, in fact, is involved in the binding of two metal ions [14]. It is therefore possible that inositol monophosphatase also possesses a similar capacity to bind two metal ions, this is currently under investigation. It has previously been suggested that inositol monophosphatase possesses two kinetically distinct metal binding sites, and these data lend themselves to the same conclusion [9]. Inositol polyphosphate 1-phosphatase has also been shown to contain the same metal-binding motif as inositol monophosphatase [23] and fructose 1,6-bisphosphatase. This enzyme is also inhibited by Li^{+} and demonstrates positive cooperative effects with respect to Mg^{2+} binding, suggesting that this enzyme may also possess dual metal binding sites per subunit.

The data presented in this paper requires alterations to the proposed [8] mechanism shown in Fig. 1. In the reverse direction, the proposed mechanism demands that P_i can bind to the enzyme in the absence of Mg^{2+} ions since the latter is shown to dissociate after the inositol but before the P_i. Our data suggests that the formation of an $E{\cdot}P_i$ complex cannot be formed. However, the simplest way of reconciling the two sets of data is to propose that in the forward direction there is the formation of an $E{\cdot}Mg^{2+}{\cdot}Mg^{2+}{\cdot}Ins(1)P$ pre-catalytic complex. Thus all subsequent intermediates through to the release of free enzyme would contain an extra Mg^{2+} ion.

Acknowledgements: We are indebted to The Wellcome Trust for the Postdoctoral fellowship for P.J G.

REFERENCES

[1] Gee, N.S., Ragan, C.I., Watling, K.J., Aspley, S., Jackson, R.G., Reid, G R., Gani, D. and Shute, J.K. (1988) Biochem. J. 249, 883–889.
[2] Hallcher, L.H. and Sherman, W.R (1980) J. Biol. Chem. 255, 10896–10901
[3] Nahorski, S.R., Ragan, C.I. and Challis, R.A.J. (1991). TIPS. 12, 297–303.
[4] Attwood, P.V., Ducep, J.B. and Chanal, N.C (1988) Biochem. J. 253, 387–394.
[5] Bone, R., Springer, J.P. and Atack, J.R (1992) Proc. Natl. Acad. Sci. USA 89, 10031–10035.
[6] Pollack, S.J., Knowles, M.R., Atack, J.R., Broughton, H.B., Ragan, C.I., Osborn, S. and McAllister, G. (1993) Eur J. Biochem. (submitted).
[7] Greasley, P.J., Gore, M.G., Ragan, C.I. and McAllister, G. (1993) Biochem. J. (submitted).
[8] Leech, A P., Baker, G.R., Shute, J.K , Cohen, M.A. and Gani, D. (1993) Eur. J Biochem 212, 693–704.
[9] Ganzhorn, A.J. and Chanal, M.C. (1990) Biochemistry 29, 6065–6071.
[10] Gee, N.S., Knowles, M.R , McAllister, G. and Ragan, C.I. (1991) FEBS Lett 284, 95–97.
[11] Knowles, M.R., Gee, N., McAllister, G., Ragan, C.I., Greasley, P J. and Gore, M.G. (1992). Biochem. J. 285, 461–468.
[12] Jackson, R.G , Gee, N S. and Ragan, C.I. (1989) Biochem. J. 264, 419–422.
[13] Zhang, Y., Liang, J.-Y and Lipscomb, W N. (1993) BBRC 190, 1080–1083.
[14] Zhang, Y., Liang, J.-Y., Huang, S., Hengming, K. and Lipscomb, W N. (1993) Biochemistry 32, 1844–1857.
[15] Greasley, P.J., Gore, M.G , Rees-Milton, K.J. and Ragan, C.I (1993) FEBS Lett. 319, 49–43.
[16] Botello, L.H., El-Dorry, H.A., Crivellaro, O., Chu, D.K., Pontremoli, S. and Horecker, B.L. (1977), Arch. Biochem. Biophys. 184, 535–545.
[17] Ke, H., Zhang, Y. and Lipscomb, W N. (1990) Proc Natl Acad. Sci. USA 87, 5243 5247.
[18] Tejwani, G.A. (1983) Adv. Enzymol. 54, 121–194.
[19] Diehl, R.E., Whiting, P , Potter, J., Gee, N., Ragan, C.I., Linemeyer, D., Schoepter, R., Bennett, C. and Dixon, R.A.F. (1990) J. Biol. Chem., 265, 5946–5949
[20] Gore, M G., Greasley, P.J. and Ragan, C I. (1992) J. Biochem. Biophys. Methods 25, 55–60.
[21] Smith, P.K., Krohn, R I., Hermanson, G.T , Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klent, D.C. (1985) Anal. Biochem. 150, 76–85.
[22] Womack, F.C. and Colowick, S.P. (1973) Methods Enzymol. 27, 464–471.
[23] Neuwald, A F., York, J. D. and Majerus, P.W. (1991) FEBS Lett. 294, 16–18.