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Method for the enzymatic synthesis of 2-phospho-D-glycerate from adenosine 5′-triphosphate and D-glycerate via D-glycerate-2-kinase

Paul A. Sims, George H. Reed*

Department of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, WI 53726, USA

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Abstract

An enzymatic synthesis of the glycolytic intermediate, 2-phospho-D-glycerate, is reported. This synthesis was undertaken as a means to make 2-phosho-D-glycerate quickly and inexpensively, because this compound is no longer available commercially. The synthesis uses the recombinant enzyme, D-glycerate-2-kinase, and the starting compounds adenosine 5′-triphosphate and D-glycerate from racemic D–L-glycerate. This enzymatic reaction is both stereospecific and regiospecific, producing 2-phospho-D-glycerate exclusively. The product is purified by anion-exchange chromatography using gradient elution with a volatile buffer. This straightforward method produces the potassium salt of 2-phosho-D-glycerate in high yield. The expression and purification of D-glycerate-2-kinase also are discussed.

Keywords: 2-phospho-D-glycerate synthesis; D-glycerate-2-kinase

1. Introduction

The compound 2-phospho-D-glycerate (2-PGA), an intermediate of glycolysis, is invaluable in the study of the enzymes that use it as a substrate, i.e., phosphoglycerate mutase and enolase. 2-PGA also is a part of a transport system in *Salmonella typhimurium* [1], and it functions as an endogenous energy source for sugar accumulation in *Streptococcus lactis* [2]. Additionally, 2-PGA is implicated in insulin secretion by its apparent stimulation of phosphorylation of a protein that is found in the pancreatic islets of rats [3]. Finally, 2-PGA was used in crystallization studies of other glycolytic enzymes [4,5]. Despite its central importance in metabolism and its utility to researchers, the chemical is no longer available commercially. This lack of commercial availability forced a search for a simple, inexpensive means of producing 2-PGA.

Three obvious methods of producing 2-PGA include (i) organic synthesis; (ii) enzymatic conversion of 3-phospho-D-glycerate (3-PGA) to 2-PGA via phosphoglycerate mutase; (iii) enzymatic conversion of phosphoenolpyruvate (PEP) to 2-PGA via enolase. The established method of organic synthesis is time-consuming and requires several steps [6]. Conversion of 3-PGA to 2-PGA via phosphoglycerate mutase has the disadvantage of an unfavorable equilibrium constant of ∼0.17 for formation of 2-PGA from 3-PGA [7,8], which is compounded by the difficulty of separating 3-PGA from 2-PGA [6]. Conversion of PEP to 2-PGA via enolase also has the disadvantage of an unfavorable equilibrium constant of ∼0.23 for formation of 2-PGA from PEP [9,10], although the separation of 2-PGA from PEP is not difficult [11].

The recent report that orf b3124 of *Escherichia coli* K-12 encodes a glycerate kinase, the gene product of which catalyzes phosphorylation of D-glycerate at the 2-position [12], suggested an alternative route, as summarized in Eq. (1)

$$\text{ATP} + \text{D-glyc} \overset{\text{Mg}^{2+}\text{,G2K}}{\rightleftharpoons} \text{ADP} + \text{2-PGA} + \text{H}^{+} \quad (1)$$

where ATP, D-glyc, and ADP represent adenosine 5′-triphosphate, D-glycerate, and adenosine 5′-diphosphate, re-

* Corresponding author. Tel.: +1 608 262 0509; fax: +1 608 265 2904.
E-mail address: reed@biochem.wisc.edu (G.H. Reed).

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spectively. G2K represents D-glycerate-2-kinase, as discussed below.

This enzymatic synthesis was tested, and it produced 2-PGA in a straightforward manner. Reported herein are the expression and purification of this glycerate kinase, as well as the protocol for using this enzyme to make 2-PGA from ATP and D-glyc. Since a previously characterized glycerate kinase from *E. coli* was found to catalyze phosphorylation of D-glyc at the 3-position and was therefore called D-glycerate 3-kinase [13], the glycerate kinase discussed in this report will be referred to as D-glycerate 2-kinase or G2K. The expression plasmid that encodes G2K will be made available to researchers who request it.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Adenosine 5′-triphosphate, disodium salt (ATP); adenosine 5′-diphosphate, monopotassium salt dihydrate (ADP); D-(+)-glyceric acid, hemicalcium salt (D-glyc); L-(−)-glyceric acid, hemicalcium salt (L-glyc); imidazole; β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH); NaN_3; phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (St. Loius, MO, USA). D–L-Glyceric acid, calcium salt (D–L-glyc) and phosphoenolpyruvate, monopotassium salt (PEP), were purchased from Alfa Aesar (Ward Hill, MA, USA). Triethylamine was purchased from Aldrich (Milwaukee, WI, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Indofine (Somerville, NJ, USA). Ampicillin, sodium salt (amp) was purchased from Amresco (Solon, OH, USA). Tris(hydroxymethyl)aminomethane (Tris); *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes); ethylenediamine tetraacetic acid, disodium salt dihydrate (EDTA); $MgCl_2$ hexahydrate; NaCl; NaH_2PO_4 were purchased from Fisher (Pittsburgh, PA, USA).

2.1.2. Chromatography materials

Activated charcoal (10–16 mesh), the anion exchange resin, AG®MP-1M, 200–400 mesh, and the cation exchange resin, AG®50W-X8, 100–200 mesh, were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ni-NTA agarose resin was purchased from Qiagen (Hilden, Germany). Sep-Pak C18 cartridges were purchased from Waters (Wexford, Ireland).

2.1.3. Enzymes

Lysozyme was purchased from Sigma. DNase I, and the coupling enzymes lactate dehydrogenase (LDH) and pyruvate kinase (PK) were purchased from Roche (Indianapolis, IN, USA). Enolase, which was used as a coupling enzyme, was purified from baker's yeast (*Saccharomyces cerevisiae*) as described previously [14]; however, enolase from a commercial vendor is suitable for use in this regard.

2.2. The pET-G2K expression vector

The gene that encodes G2K was amplified by PCR from *E. coli* K-12 genomic DNA using the following primers from Integrated DNA Technologies (IDT) (Coralville, IA, USA):

forward : 5′-CACCATGGCGTATTGCAATCCGG-3′

reverse : 5′-CGCGTTGCGCATTCCAATCG-3′

These primers were designed according to the guidelines in the Champion™ pET Directional TOPO® Expression Kit from Invitrogen (Carlsbad, CA, USA). This kit uses a topoisomerase I-based method of directional cloning [15] to insert the PCR product directly into the expression vector; thus the primers contain no restriction sites. The first four bases of the forward primer were added to impart directionality for the subsequent cloning into the pET101/Directional-TOPO® vector. The reverse primer was designed such that the C-terminal glycine was removed, to reduce the GC content of the primer. In addition, the reverse primer also was designed to read in-frame (i.e., without a stop codon) with the C-terminal fusion tag of the above vector. This fusion tag consisted of the V5 epitope followed by six histidine residues. The resulting expression vector was sequenced at the University of Wisconsin-Madison Biotechnology Center and named pET-G2K.

2.3. Expression and purification of G2K

An aliquot of pET-G2K DNA is used to transform BL21 (DE3) cells according to the cell supplier's recommendations (cells from Invitrogen generally are used). The transformation reaction is spread on an LB plate that contains 0.4 g L^{-1} amp and incubated overnight at 37 °C. A single colony from the overnight growth is transferred to a 15 mL culture tube that contains 5 mL of LB plus 0.4 g L^{-1} amp. This culture is grown at 37 °C for 4 h and then is diluted into 2 L flasks (0.75 mL culture per 2 L flask), each of which contain 1 L of LB plus 0.4 g L^{-1} amp. Prior to dilution, the A_{600} of the 5 mL culture, when corrected for dilution, is ∼1. The diluted cultures are shaken (200 rpm) overnight at 37 °C and then transferred to room temperature where they are shaken at a slower rate of 150 rpm. When this transfer to room temperature occurs, the A_{600} of the cultures, when corrected for dilution, is ∼4. Expression of G2K is induced by addition of 0.2 g L^{-1} IPTG to each flask. Approximately 7 h after addition of IPTG, cells are harvested by centrifugation, frozen in liquid nitrogen, and stored at −20 °C.

Cells from the above growth (∼25 g) are added to 250 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8.0), which contains 140 mg lysozyme, 40 mg PMSF, and ∼1 mg DNase I. The lysis mixture is gently

shaken (∼130 rpm) at room temperature for 2 h and then is centrifuged at 6000 RCF and at 4 °C for 20 min. The resulting supernatant is taken into a ∼4 °C room and loaded slowly onto a 2.5 cm × 3.5 cm column of Ni-NTA agarose, which has been equilibrated in lysis buffer. After loading of the sample, the resin is washed with 400 mL of wash buffer before the elution buffer is applied. (The wash and elution buffers are the same as the lysis buffer but have 20 and 250 mM imidazole, respectively.)

The flow-through of the elution buffer is collected in 12 mL fractions. Samples from these fractions can be analyzed by SDS-PAGE and/or assayed for activity as discussed below. Those fractions that contain G2K are pooled, exchanged into storage buffer (20 mM Tris, pH 7.5; 2 mM $MgCl_2$; 1 mM NaN_3; 0.5 mM EDTA), filtered through a 0.22 μM syringe filter, and then frozen with dry ice and stored at −80 °C. Concentrations of G2K are estimated from the calculated $\varepsilon_{280}^{1\%}$ of 4.3 [16]. This protein expression and purification protocol generally yields ∼100 mg G2K L^{-1} of culture.

The assay for G2K activity [17] is run at 25 °C and includes, in a 1 mL volume, the following components: 50 mM Hepes/KOH, pH 7.5; ∼0.25 mM NADH; ∼0.5 mM ATP; 0.20 mM D-glyc, hemicalcium salt; 2.5 mM $MgCl_2$; 14 U LDH; 10 U PK; 15 U enolase; 10 μL of the G2K sample. The above components, minus enzymes, are combined, and the A_{340} is monitored. Coupling enzymes are added separately in the following order: LDH, PK, and enolase. After each coupling enzyme is added, the A_{340} is checked to note any possible changes. When the A_{340} is steady, the next coupling enzyme is added. G2K is added last and any decrease in A_{340} (due to NADH oxidation) may be attributed to its activity. A representative assay is shown in Fig. 1.

Assays to determine the specific activity of G2K are run with the potassium salt of D-glyc to avoid inhibition of the coupling enzymes by calcium. Under these conditions, G2K shows a specific activity of 80 U mg^{-1}. (The hemicalcium salt of D-glyc – the only type available commercially – is converted into the potassium salt in an analogous manner to that which is described below for conversion of the triethylammonium salt of 2-PGA to the potassium salt.)

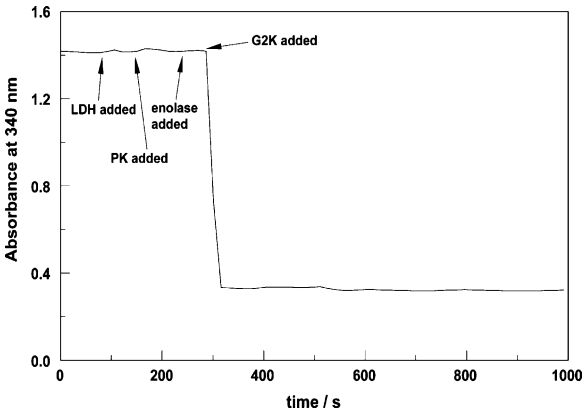


Fig. 1. Assay for G2K activity. The absorbance at 340 nm remains relatively constant after the addition of LDH, PK, and enolase. When G2K is added, the absorbance decreases due to the G2K-catalyzed conversion of D-glyc and ATP to 2-PGA and ADP, which leads, ultimately, to the oxidation of NADH.

2.4. Enzymatic synthesis of 2-PGA

ATP (4.3 g) and D–L-glyc (1.1 g) are dissolved in ∼35 mL H_2O. The pH of this solution is adjusted to 7–8 by slow addition of concentrated KOH, because potassium ions were shown to activate G2K from *Hyphomicrobium methylovorum* [17]. G2K (10 mg) and $MgCl_2$ (2 mM final) are added to the neutralized solution, and the reaction proceeds at room temperature overnight.

2.5. Purification of 2-PGA

2.5.1. Pre-chromatography

The following day, the reaction mixture is prepared for anion exchange chromatography by passing it repeatedly through a 2.5 cm × 12 cm column of activated charcoal until the A_{260}, when corrected for dilution, is ∼6. This pre-reduction of nucleotides prevents the anion exchange column, which is used in the next step, from becoming overloaded with nucleotides to the exclusion of 2-PGA.

2.5.2. Anion exchange chromatography

After repeated passage through the charcoal column, the reaction mixture is diluted to ∼0.2 L and loaded at 2.5 mL min^{-1} onto a 2 cm × 20 cm column of AG®MP-1 M resin, which is in the HCO_3^- form. The loaded column is then washed with a similar volume of H_2O, but at 5 mL min^{-1}. (The AG®MP-1 M resin, which is sold dry and in the Cl^- form, is first made into a slurry by addition of H_2O; this slurry is poured into the column, and the resin is converted to the HCO_3^- form by passing 1.8 L of 1.5 M ammonium bicarbonate through the column. The column is then washed with the same volume of H_2O.)

Once the column has been washed, 2-PGA is eluted from the column with a 0.5 L gradient from 0.25 to 0.5 M triethylammonium bicarbonate (TEAB) buffer. This volatile buffer, which is used to avoid extraneous salt in the final product, is made by diluting freshly distilled triethylamine into H_2O, followed by addition of CO_2 to saturation via a gas dispersion tube. When fully saturated with CO_2, the buffer is ready and needs to be used promptly. Another volatile buffer (e.g., ammonium bicarbonate) might be suitable for this purification, but no others were tested.

The anion-exchange chromatography is carried out with a Pharmacia FPLC; however, another pressure-chromatography system could be substituted. If a pressure-chromatography system is unavailable, other chromatographic options are feasible [18,19].

Fractions from the anion-exchange column are checked for the presence of 2-PGA by a coupled assay [20]. The assay cocktail includes all coupling enzymes and is used repeatedly until the ADP and/or NADH are exhausted. In this manner,

all of the fractions can be assayed relatively rapidly. The purification may, if desired, be stopped at this point, and the fractions that contain 2-PGA can be stored overnight at 4 °C.

2.5.3. Concentration of 2-PGA

The fractions that contain 2-PGA are pooled, and the solvent is removed under reduced pressure with a rotary evaporator until a small volume of clear syrup is obtained. This syrup is dissolved in ∼15 mL of methanol, and the volume of the resulting solution is again reduced with the rotary evaporator. This process is repeated approximately eight times until the odor of triethylamine is no longer apparent in the flask.

2.5.4. Conversion to the potassium salt of 2-PGA

The washed syrup from step 8, which contains the triethylammonium salt of 2-PGA, is dissolved in ∼10 mL of H_2O and added to the top of a 2 cm × 25 cm column of the cation-exchange resin, AG®50W-X8, which is in the H^+ form. The flask that contained the 2-PGA syrup is washed with H_2O, and this wash is added to the column. The column is then eluted with H_2O. Fractions that contain 2-PGA are acidic and can be detected with pH paper. These fractions are pooled and immediately neutralized with concentrated KOH.

The neutralized 2-PGA solution may be slightly colored from the dye in the AG®50W-X8 resin; if so, the solution may be decolorized by passage through a Sep-Pak C-18 cartridge. Finally, the 2-PGA solution is shell-frozen and lyophilized. The product is stored in a desiccator at −20 °C.

3. Results and discussion

3.1. Properties of G2K and the reaction product

G2K readily expresses in BL21 (DE3) cells upon induction with IPTG. The C-terminal His-tag allows for a simple, one-step purification with a Ni-NTA agarose column. Despite having six cysteine residues, G2K does not require dithiothreitol to retain full activity. G2K also retains full activity through at least one freeze–thaw cycle and through prolonged storage at 4 °C, provided the storage buffer contains 1 mM NaN_3.

As mentioned, different glycerate kinases exhibit different regiospecificities. Because the earliest characterizations of various glycerate kinases did not always definitively show which product was formed, confusion persists concerning the identity of the reaction product (see, for example, http://www.brenda.uni-koeln.de/). The glycerate kinase discussed in this report (G2K) catalyzes phosphorylation of D-glyc at the 2-position exclusively. The possibility that G2K co-purifies with phosphoglycerate mutase is ruled out by the observation that the ^{1}H NMR spectrum of authentic 2-PGA obtained from Sigma (when it was available) is identical with that produced by the above synthesis (Fig. 2). This result is consistent with a previous report [12], in which the ^{1}H NMR spectra of 3-PGA and 2-PGA were compared with that of

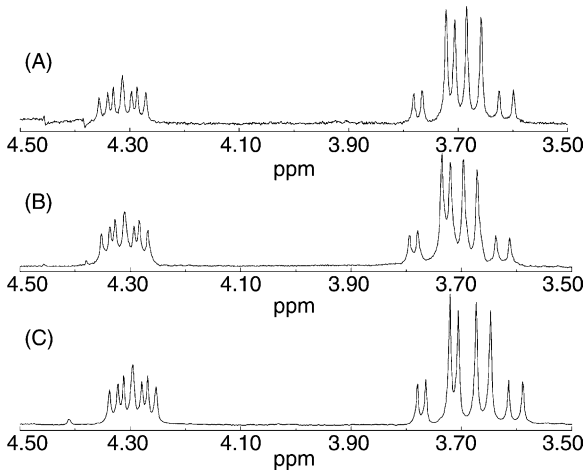


Fig. 2. (A) ^{1}H NMR spectrum of authentic 2-PGA from Sigma; (B) ^{1}H NMR spectrum of 2-PGA produced via the G2K-catalyzed phosphorylation of D-glyc; (C) ^{1}H NMR spectrum of equimolar mixture of 2-PGA from Sigma and from the synthesis. Slight differences in peak positions seen in panels (A) and (B) are due to differences in pD. The equimolar mixture in panel (C) results in a single pD and shows that the two samples contain the same compound. All spectra were obtained with a 200 MHz NMR spectrometer.

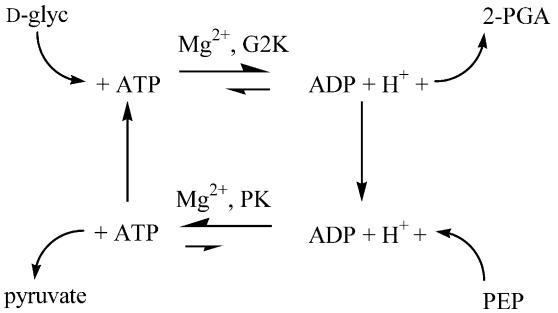
the reaction mixture. If any phosphoglycerate mutase were present, an equilibrium mixture of 3-PGA and 2-PGA would result, with the equilibrium favoring 3-PGA. Thus, the regiospecificity of the reaction is confirmed.

The G2K-catalyzed reaction is alsostereospecific, producing only 2-phospho-D-glycerate. Assays with L-glyc and G2K showed no detectable activity with this epimer (not shown). Thus, the less expensive, racemic D–L-glyc may be used for the synthesis of 2-PGA without loss of stereochemical fidelity in the product.

3.2. The enzymatic synthesis

The above synthesis produces 2-PGA with an overall yield of ∼90% (based on the D-glyc content of racemic D–L-glyc). The amounts of D–L-glyc and ATP given in the protocol will yield ∼0.5 g of 2-PGA. These amounts correspond to a 2:1 mole ratio of ATP to D-glyc; the excess ATP helps to buffer the reaction mixture, although a decrease in pH of ∼1 unit is typical during a synthesis reaction.

A variation of the above synthesis that uses PEP and PK to regenerate ATP from ADP was also tested, as summarized in Eq. (2)



(2)

This variation uses only a catalytic amount of ATP, and the pH of the system remains constant. The starting [PEP] is at 95% of the starting [D-glyc] to avoid having to separate 2-PGA from PEP. Use of this variation obviates the need to pass the reaction mixture through a charcoal column prior to anion-exchange chromatography. A slightly different elution of 2-PGA from the anion-exchange resin is also required, where a ∼125 mL wash at 0.25 M TEAB removes most of the pyruvate prior to the start of the gradient. Detecting 2-PGA in the post-anion-exchange fractions is more involved, as the fractions that contain pyruvate must be distinguished from those that contain 2-PGA. The percent yield that is obtained with this ATP-regenerating procedure is similar to that which is obtained with the ATP-consuming method, but the equilibrium point in the ATP-regenerating method takes longer to reach (∼2 days). This delay in reaching equilibrium is likely due to inhibition of PK by Ca^{2+} (since the calcium salt of D–L-glyc is used) [21]. In addition, the PK must be checked to ensure that it is not contaminated with enolase.

4. Conclusion

A straightforward method to make 2-PGA quickly and inexpensively is presented. This method will alleviate the problem of not having commercial sources for this important metabolite. The method requires a minimum of specialized equipment. In addition, this method requires only two starting compounds, ATP and D–L-glyc, and one enzyme, G2K. Both starting compounds are readily available commercially, and the G2K enzyme, as mentioned, will be made available (in the form of an expression plasmid) to researchers who request it. The product of the synthesis is purified in one step via anion-exchange chromatography, which is followed by concentration of the product and conversion of the product to the potassium salt. A second method to make 2-PGA, in which ATP is regenerated from ADP, also is discussed. This ATP-regenerating method requires an additional starting compound, PEP, and an additional enzyme, PK, but it works as well as the ATP-consuming method and represents another option for making 2-PGA with G2K.

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References

[1] M.H. Saier Jr., D.L. Wentzel, B.U. Feucht, J.J. Judice, J. Biol. Chem. 250 (1975) 5089.
[2] J. Thompson, T.D. Thomas, J. Bacteriol. 130 (1977) 583.
[3] S.B. Pek, M. Usami, N. Bilir, C. Fischer-Bovenkerk, T. Ueda, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4294.
[4] M.E. Noble, C.L. Verlinde, H. Groendijk, K.H. Kalk, R.K. Wierenga, W.G. Hol, J. Med. Chem. 34 (1991) 2709.
[5] S. Parthasarathy, K. Eaazhisai, H. Balaram, P. Balaram, M.R. Murthy, J. Biol. Chem. 278 (2003) 52461.
[6] C.E. Ballou, H.O.L. Fischer, J. Am. Chem. Soc. 76 (1954) 3188.
[7] O. Meyerhof, P. Oesper, J. Biol. Chem. 179 (1949) 1371.
[8] V.W. Rodwell, J.C. Towne, S. Grisolia, J. Biol. Chem. 228 (1957) 875.
[9] F. Wold, C.E. Ballou, J. Biol. Chem. 227 (1957) 301.
[10] J.J. Burbaum, J.R. Knowles, Biochemistry 28 (1989) 9306.
[11] T.Y.S. Shen, E.W. Westhead, Biochemistry 17 (1973) 3333.
[12] B.K. Hubbard, M. Koch, D.R. Palmer, P.C. Babbitt, J.A. Gerlt, Biochemistry 37 (1998) 14369.
[13] C.C. Doughty, J.A. Hayashi, H.L. Guenther, J. Biol. Chem. 241 (1966) 568.
[14] J.E. Wedekind, R.R. Poyner, G.H. Reed, I. Rayment, Biochemistry 33 (1994) 9333.
[15] S. Shuman, J. Biol. Chem. 269 (1994) 32678.
[16] S.C. Gill, P.H. von Hippel, Anal. Biochem. 182 (1989) 319.
[17] T. Yoshida, K. Fukuta, T. Mitsunaga, H. Yamada, Y. Izumi, Eur. J. Biochem. 210 (1992) 849.
[18] V.E. Anderson, P.M. Weiss, W.W. Cleland, Biochemistry 23 (1984) 2779.
[19] G.H. Reed, T.S. Leyh, Biochemistry 19 (1980) 5472.
[20] F. Wold, Enzymes 5 (1971) 499.
[21] J.F. Kachmar, P.D. Boyer, J. Biol. Chem. 200 (1953) 669.