Biochimica et Biophysica Acta, 660 (1981) 193–198
Elsevier/North-Holland Biomedical Press

BBA 69323

EVIDENCE FOR ABSENCE OF AN INTERACTION BETWEEN PURIFIED 3-PHOSPHOGLYCERATE KINASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

MÁRIA VAS and JÓZSEF BATKE

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, P O Box 7 (Hungary)

(Received, 19th February, 1981)

Key words Enzyme-enzyme interaction, 3-Phosphoglycerate kinase, Glyceraldehyde-3-phosphate dehydrogenase

The possibility of a functional complex formation between glyceraldehyde-3-phosphate dehydrogenase (EC 1 2 1 12) and 3-phosphoglycerate kinase (EC 2 7 2 3), enzymes catalysing two consecutive reactions in glycolysis has been investigated Kinetic analysis of the coupled enzymatic reaction did not reveal any kinetic sign of the assumed interaction up to 4 10^{-6} M kinase and 10^{-4} M dehydrogenase Fluorescence anisotropy of 10^{-7} M or 2 10^{-5} M glyceraldehyde-3-phosphate dehydrogenase labeled with fluorescein isothiocynate did not change in the presence of non-labeled 3-phosphoglycerate kinase (up to 4 10^{-5} M) The frontal gel chromatographic analysis of a mixture of the two enzymes (10^{-4} M dehydrogenase and 10^{-5} M kinase) could not reveal any molecular species with the kinase activity having a molecular weight higher than that of 3-phosphoglycerate kinase Both types of physicochemical measurements were also performed in the presence of substrates of the kinase and gave the same results The data seem to invalidate the hypothesis that there is a complex between purified pig muscle glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase

Introduction

The relative proportions of most glycolytic enzymes have been demonstrated to be nearly constant in a wide variety of tissues [1,2] Also taking into account their high physiological concentration [1,3], the hypothesis of specific complex formation between the enzymes of this group has been put forward [4] In fact, the structural organization of certain glycolytic enzymes, both on the surface of various cellular structures (Ref 5 and references therein and Refs 6 and 7) and even without any supporting matrix [8–11] has been detected

The possibility of specific interaction of glyceraldehyde-3-phosphate dehydrogenase (EC 1 2 1 12) with 3-phosphoglycerate kinase (EC 2 7 2 3) has also been postulated [12] on the following grounds Both enzymes belong to the constant proportion group of glycolytic enzymes [1,2] The asymmetrically shaped monomeric molecule of 3-phosphoglycerate kinase consists of two large domains [13] It has been suggested that one of these domains might have some role in complex formation with glyceraldehyde-3-phosphate dehydrogenase [12] Furthermore, speculations about a unique three-dimensional structure including the two enzymes have been put forward [4]

In view of these suggestions we investigated the possibility of complex formation between glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase This paper presents kinetic and physicochemical (fluorescence anisotropy and gel chromatographic) studies on the purified pig skeletal muscle enzymes at concentrations near to the estimated [1,3] physiological range (10^{-5}–10^{-4} M)

Materials and Methods

Glyceraldehyde-3-phosphate dehydrogenase containing tightly-bound NAD (an average of 3 5 mol/

0005-2744/81/0000–0000/$02 50

tetrameric enzyme) [14] and 3-phosphoglycerate kinase [15] were prepared from pig muscle and stored as crystal suspensions at 4°C Before the experiments the crystalline enzymes were dissolved in 0 1 M triethanolamine-HCl buffer, pH 7 5/5 mM mercaptoethanol and gel-filtered on a Sephadex G-50 column equilibrated with the same buffer to remove $(NH_4)_2SO_4$

Enzyme concentrations were determined spectrophotometrically by using $A_{280}^{0.1\%} = 1\ 0$ and 0 69 for glyceraldehyde-3-phosphate dehydrogenase [16] and 3-phosphoglycerate kinase [15], respectively The specific activity of the dehydrogenase calculated for the tetramer varied between 15 000 and 18 000 M substrate/M enzyme min^{-1} at 20°C in the reaction with 2 10^{-4} M NADH and 10^{-3} M 1,3-diphosphoglycerate in 0 1 M triethanolamine-HCl buffer/5 mM mercaptoethanol The same value was obtained if 1,3-diphosphoglycerate was generated in the reaction mixture by 3-phosphoglycerate kinase from 1 5 10^{-2} M 3-phosphoglycerate, 10^{-2} M ATP and 10^{-2} M $MgCl_2$ Under the same conditions the specific activity of 3-phosphoglycerate kinase varied between 35 000 and 38 000 M substrate/M enzyme min^{-1} in the reaction mixture containing 1 5 10^{-2} M 3-phosphoglycerate, 10^{-2} M ATP, 10^{-2} M $MgCl_2$, 2 10^{-4} M NADH and about 100-fold molar excess of glyceraldehyde-3-phosphate dehydrogenase over the kinase The molecular weights of the tetrameric dehydrogenase and the monomeric kinase were taken to be 145 000 [17] and 48 000 [12], respectively

3-Phosphoglycerate and fluorescein isothiocyanate were Boehringer and Calbiochem products, respectively, ATP and NADH were Reanal preparations 1,3-Diphosphoglycerate was prepared from glyceraldehyde 3-phosphate according to the method of Furfine and Velick [18] Glyceraldehyde 3-phosphate was prepared from fructose 1,6-diphosphate, as described by Szewczuk et al [19] All other chemicals were reagent grade commercial preparations The concentrations of all substrate solutions were determined enzymatically

Kinetic measurements

Kinetic studies were carried out with a Cary 118 recording spectrophotometer and with a Durrum-Gibson stopped-flow apparatus (Model 110) The temperature of the cuvette house and the reaction chamber was kept at 20°C

The consecutive enzymatic reactions starting from 3-phosphoglycerate, ATP, $MgCl_2$ and NADH catalyzed by glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase were followed at 366 nm ($\epsilon_{366}^{NADH} = 3\ 3\ \ 10^3\ M^{-1}\ cm^{-1}$ [20]) Occasionally, the coupled reaction was studied in the absence of NADH on the basis of the disappearence of the characteristic Racker band of the glyceraldehyde-3-phosphate dehydrogenase NAD complex ($\epsilon_{360} = 800\ M^{-1}\ cm^{-1}$ [21]) due to the acylation of the dehydrogenase with 1,3-diphosphoglycerate generated by the kinase reaction [22] Reaction mixtures always contained hidrazine hydrate to trap the product (glyceraldehyde 3-phosphate), thereby making the reactions practically irreversible [23] Routinely the mixture of all substrates containing both hidrazine and the mixture of the two enzymes were introduced separately into the two syringes of the stopped-flow instrument The dead time of the instrument was about 2–5 ms

Fluorescence labeling and measurement

2 ml of 2 10^{-4} M glyceraldehyde-3-phosphate dehydrogenase were labeled with 5 mg fluorescein isothiocyanate (on celite carrier) in 0 1 M triethanolamine-HCl buffer, pH 8 5 The mixture was incubated for 1 h in the dark at 5°C Free fluorescent dye was then removed by gel filtration through a column of Sephadex G-50 equilibrated with 0 1 M triethanolamine-HCl buffer, pH 7 5, containing 5 mM mercaptoethanol The extent of the labeling was determined on the basis of absorbance of the treated enzyme at 490 nm ($\epsilon_{490} = 3\ 4\ \ 10^4\ M^{-1}\ cm^{-1}$) [24] To calculate the concentration of the labeled enzyme the absorbance of the dye at 280 nm ($\epsilon_{280} = 1\ 09\ \ 10^4\ M^{-1}\ cm^{-1}$) was taken into account The degree of the labeling varied between 0 5–1 0 mol dye/mol tetrameric enzyme The labeled enzyme retained 90–95% of its original specific activity

Fluorescence measurements were carried out in an SP 3 spectrofluorimeter (Applied Photophysics Ltd), in a 10 × 10 mm cell, thermostatically maintained at 20°C The excitation and emission wavelengths were 470 and 520 nm, respectively The polarization (p) and the anisotropy (A) of fluorescence were calcu-

lated according to the formulas [25]

$$p = \frac{I_{VV} - I_{VH}(G)}{I_{VV} + I_{VH}(G)}, \qquad G = \frac{I_{HV}}{I_{HH}} \qquad \text{and } A = \frac{3p}{3 - p}$$

where I is the intensity of the emitted light The first and the second indices refer to the positions (vertical, V or horizontal, H) of the polarizer and analyzer, respectively G is the instrumental factor [26]

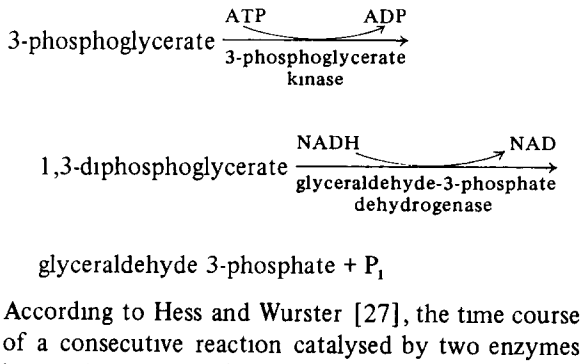
Frontal analysis gel chromatography

Ascending gel chromatographic runs were made at 10°C on Sephadex G-200 columns (0 66 × 96 cm) equilibrated with 0 1 M triethanolamine-HCl buffer, pH 7 5/5 mM mercaptoethanol The columns were loaded with a large volume (about 30 ml) of enzyme solution in the same buffer A constant flow rate of 1 2 ml/h was maintained by means of an LKB Multiperpex 2115 type peristaltic pump Fraction volume was 0 5 ml Effluent fractions were assayed for enzyme activities The plateau activity of each enzyme attained that of the loading enzyme solutions

Results and Discussion

Kinetics of the consecutive reactions catalysed by 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase

The coupled reaction described by the scheme below was studied by applying saturating concentrations of substrates The dehydrogenase was used in 20-fold molar excess over the kinase, i e , its activity in the reaction mixture was about 10-times as high as the activity of the kinase



According to Hess and Wurster [27], the time course of a consecutive reaction catalysed by two enzymes has a lag period (characterized by the transient time, τ) before reaching the steady-state velocity (v_{ss}), both values being related to the activities of the two enzymes In our system, v_{ss} is limited by the activity of the kinase and the transient time is inversely proportional to the activity of the dehydrogenase If the assumed enzyme-enzyme interaction between the kinase and the dehydrogenase has some kinetic consequences, some kind of deviation from either of these relations should be observed with increasing enzyme concentrations

It should be noted that in the absence of kinase the activity of glyceraldehyde-3-phosphate dehydrogenase is proportional to its concentration [28] and there is no indication of self-association of the monomeric 3-phosphoglycerate kinase molecule [12] in the absence of dehydrogenase Therefore, any deviation from linearity can only be ascribed to interaction between the two enzymes Thus, keeping the molar ratio between the two enzymes constant, we increased the concentration of both enzymes and measured the consecutive reaction The steady-state velocities and the reciprocal transient times were plotted against the kinase and dehydrogenase concentrations (Fig 1A and B), respectively

In some cases the coupled reaction was studied in the absence of NADH In this case the reaction of glyceraldehyde-3-phosphate dehydrogenase does not reach completion, acyl-enzyme, produced by reaction with 1,3-diphosphoglycerate, is the end product There is an indication [29] that this covalent intermediate of the reaction of glyceraldehyde-3-phosphate dehydrogenase is the physiologically relevant enzyme form Since the formation of 3-phosphoglyceroyl enzyme is a very fast process [30], under our experimental conditions the transient time of the coupled reaction is too small to be determined However, the steady-state velocity could be measured and practically the same values were obtained as in the presence of NADH (Fig 1A)

In none of the above experiments did any deviation from linearity appear Thus, we did not find any kinetic indication of the assumed interaction up to $4\ 10^{-6}$ M 3-phosphoglycerate kinase and 10^{-4} M glyceraldehyde-3-phosphate dehydrogenase

Fluorescence studies

Fluorescence anisotropy measurements are widely applied for studying macromolecular interactions

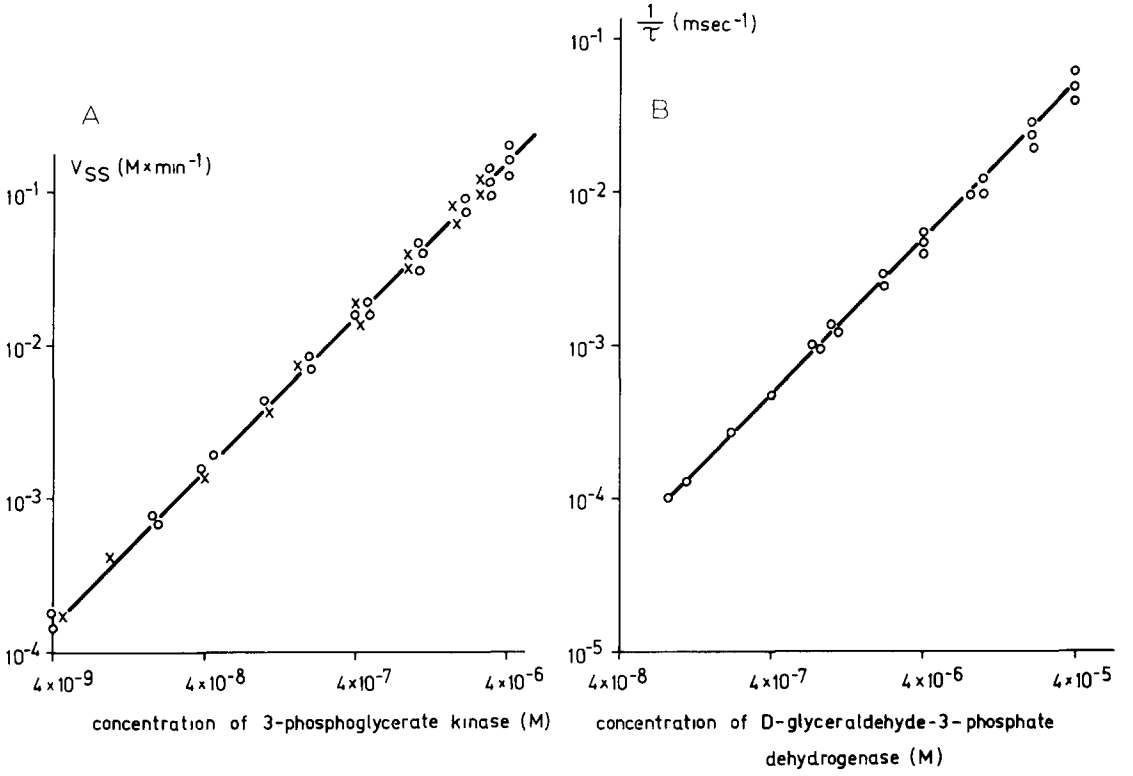


Fig 1 Kinetic analysis of the consecutive reaction catalysed by 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase Kinetics of the coupled enzymatic reaction were studied at 20°C in 0 1 M triethanolamine-HCl buffer, pH 7 5, starting from 20 mM 3-phosphoglycerate, 20 mM ATP and 20 mM $MgCl_2$ Reaction mixtures also contained 0 1 M hidrazine hydrate and 5 mM mercaptoethanol Time course of the reaction was followed either in the presence of 3 10^{-4} M NADH at 366 nm (o———o) or in the absence of NADH at 360 nm by the disappearence of the Racker band of dehydrogenase NAD complex (X———X) In the former case (o———o) the molar ratio of dehydrogenase to kinase was always 20 In the latter case (X———X) a constant dehydrogenase concentration of 10^{-4} M was used together with various kinase concentrations as indicated on the abscissa The steady-state velocity (v_{ss}) was calculated from the linear part of the time course reached after the lag period and was plotted against the kinase concentration (A) The reciprocal transient time (τ) was plotted against the dehydrogenase-concentration (B) The straight lines correspond to specific activities of 37 000 M substrate/M kinase min^{-1} and 17 000 M substrate/M dehydrogenase min^{-1} in cases A and B, respectively

[25] To detect a possible interaction between 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase we carried out fluorescence anisotropy measurements at much higher kinase concentrations than those used in the kinetic measurements

The anisotropy of fluorescence of labeled dehydrogenase was measured in the presence of increasing concentration of unlabeled kinase It was found that even 4 10^{-5} M 3-phosphoglycerate kinase did not cause any change of the anisotropy of 2 10^{-5} M glyceraldehyde-3-phosphate dehydrogenase, either in the absence or in the presence of the substrates of kinase The value of 0 39 ± 0 03 for anisotropy is characteristic of the tetrameric dehydrogenase, since at 2 10^{-5} M concentration this oligomeric form predominates [31] Thus, the results argue against complex formation between the tetrameric glyceraldehyde-3-phosphate dehydrogenase and the monomeric glyceraldehyde-3-phosphate dehydrogenase

It is known that tetrameric glyceraldehyde-3-phosphate dehydrogenase can dissociate into dimers and monomers upon dilution [31,32] In agreement with the earlier reported data on rabbit muscle glyceral-

dehyde-3-phosphate dehydrogenase [9], the dissociation is also reflected by the decrease of fluorescence polarization of the labeled pig muscle dehydrogenase (Batke, J , unpublished data) According to these observations the dehydrogenase is fully dissociated below 10^{-7} M Thus, interactions between dimeric and/or monomeric dehydrogenase and kinase may also occur To investigate this possibility, 4 10^{-5} M 3-phosphoglycerate kinase was added to the solution of dilute (10^{-7} M) labeled glyceraldehyde-3-phosphate dehydrogenase Again, kinase did not cause any change in the anisotropy of dehydrogenase (its value was 0 08 ± 0 03), which also indicates the absence of interaction

It should be noted that both experiments gave the same result even after 24 h incubation of the enzyme mixtures

Frontal analysis gel chromatography

This is particularly suitable for the study of interacting macromolecular systems [33] We employed this technique as an independent method to investigate the possibility of complex formation between 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase As seen in Fig 2, the elution volume of 10^{-5} M kinase, which corresponds to the centre of the boundary, was exactly the same both in the absence and presence of 10^{-4} M dehydrogenase The same result was obtained if 3-phosphoglycerate and ATP were added, producing aceylated glyceraldehyde-3-phosphate dehydrogenase, i e , when both enzymes were present in their functional forms Accordingly, under our experimental conditions 3-phosphoglycerate kinase does not associate, to any detectable extent, with glyceraldehyde-3-phosphate dehydrogenase

Conclusions

The above data do not support the hypothesis of a functional interaction between isolated pig muscle glyceraldehyde-3-phosphate dehydrogenase and

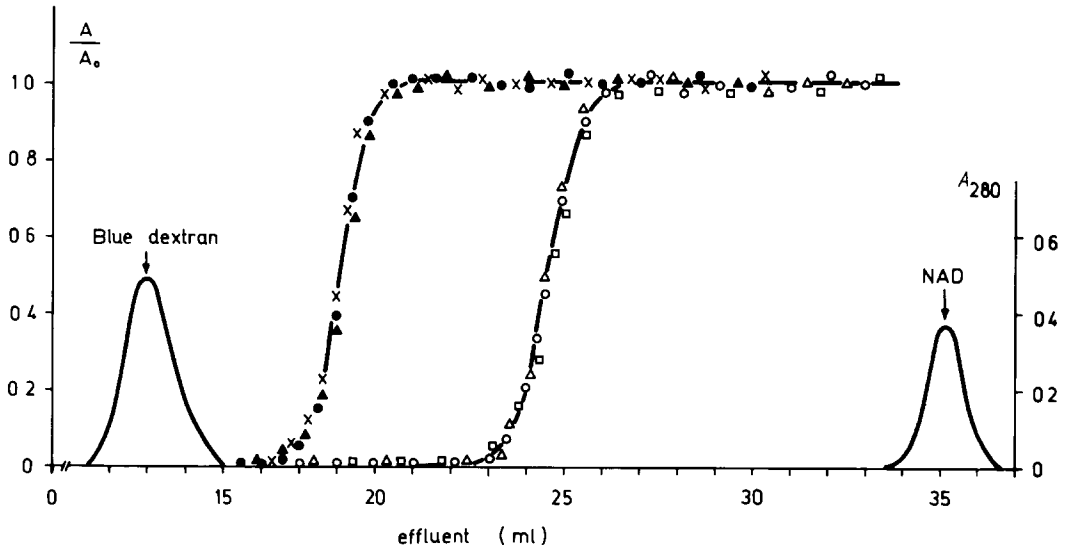


Fig 2 Frontal analysis gel chromatography of the mixture of 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase The mixture of 10^{-5} M kinase (○———○) and 10^{-4} M dehydrogenase (●———●) was loaded onto the column (see Materials and Methods) equilibrated with 0 1 M triethanolamine-HCl buffer, pH 7 5, containing 5 mM mercaptoethanol In a separate experiment the gel chromatography of a mixture consisting of the above concentrations of kinase (△———△) and dehydrogenase (▲———▲) was carried out in the presence of 20 mM 3-phosphoglycerate, 20 mM ATP and 20 mM $MgCl_2$ The column buffer also contained the substrate mixture In control experiments 10^{-4} M dehydrogenase (X———X) and 10^{-5} M kinase (□———□) were gel chromatographed separately A_0 means the enzyme activity of the solution loaded onto the column, A marks the enzyme activity of effluent fractions Blue Dextran 2 000 and NAD, applied in a volume of 0 5 ml, were run in a separate experiment and detected by absorbance at 280 nm The elution volumes for dehydrogenase and kinase were 19 2 ± 0 3 ml and 24 5 ± 0 3 ml, respectively

3-phosphoglycerate kinase On the basis of the highest enzyme concentrations used in the present work and considering the limits of error of the techniques used, the estimated dissociation of an assumed 3-phosphoglycerate kinase glyceraldehyde-3-phosphate dehydrogenase complex should be $K_d \geqslant 10^{-2}$ M

Acknowledgment

We are grateful to Mrs M Szentirmai for her excellent technical assistance

References

1 Czok, R and Bucher, T (1960) Adv Protein Chem 15, 315–415
2 Pette, D , Luh, W and Bucher, T (1962) Biochim Biophys Res Commun 7, 419–424
3 Hess, B and Boiteux, A (1972) in Protein-protein Interaction, Proceedings of 23th Moschbach Colloquium, Springer Verlag, Berlin
4 Ottaway, J H and Mowbray, J (1977) Curr Top Cell Regul 12, 155–158
5 Clarke, F M and Masters, C J (1976) Int J Biochem 7, 359–365
6 Opperdoes, F R and Borst, P (1977) FEBS Lett 80, 360–364
7 Knull, H R (1978) Biochim Biophys Acta 522, 1–9
8 Gorringe, D M and Moses, V (1980) Int J Biol Macromol 2, 161–173
9 Ovádi, J , Salerno, C , Keleti, T and Fasella, P (1978) Eur J Biochem 90, 499–503
10 Grazi, E and Trombretta, G (1980) Eur J Biochem 107, 369–373
11 Batke, J , Asbóth, G , Lakatos, S , Schmitt, B and Cohen, R (1980) Eur J Biochem 107, 389–394
12 Scopes, R K (1973) in The Enzymes, 3rd edn , (Boyer, P D ed), Vol 8 pp 335–351, Academic Press, New York
13 Blake, C C F and Evans, P R (1974) J Mol Biol 84, 585–601
14 Elodi, P and Szorényi, I (1956) Acta Phys Acad Sci Hung 9, 339–350
15 Krietsch, W K G and Bucher, T (1970) Eur J Biochem 17, 568–580
16 Fox, I B and Dandliker, W B (1956) J Biol Chem 221, 1005–1017
17 Elodi, P (1958) Acta Phys Acad Sci Hung 13, 199–206
18 Furfine, C S and Velick, S F (1965) J Biol Chem 240, 844–855
19 Szewczuk, A , Wolny, E , Wolny, M and Baranowsky, T (1961) Acta Biochim Pol 8, 210–207
20 Seydoux, F J , Kelemen, N , Kellershohn, N and Roucous, C (1976) Eur J Biochem 64, 481–489
21 Cseke, E and Boross, L (1967) Acta Biochim Biophys Acad Sci Hung 2, 39–46
22 Harrigan, P J and Trentham, D R (1973) Biochem J 135, 695–703
23 Cseke, E , Váradi, A , Szabolcsi, G and Biszku, E (1978) FEBS Lett 96, 15–18
24 Churchich, J E (1967) Biochim Biophys Acta 147, 511–517
25 Pesce, A J , Rosen, C G and Pasby, T L (1971) Fluorescence Spectroscopy, Ch 4, pp 87–130, Marcel Decker Inc , New York
26 Rank, D H (1947) Anal Chem 19, 766–767
27 Hess, B and Wurster, B (1970) FEBS Lett 9, 73–79
28 Vas, M , Lakatos, S , Hajdu, J and Friedrich, P (1981) Biochimie 63, 89–96
29 Block, W , MacQuarrie, R A and Bernhard, S A (1971) J Biol Chem 246, 780–790
30 Trentham, D R (1981) Biochem J 122, 71–77
31 Hoagland, V D and Teller, D C (1969) Biochemistry 8, 594–602
32 Lakatos, S , Závodszky, P and Elodi, P (1972) FEBS Lett 20, 324–326
33 Ackers, G K , Thompson, T E (1965) Proc Natl Acad Sci U S A 53, 342–349