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NEGATIVE HOMOTROPIC COOPERATIVITY IN RAT MUSCLE AMP DEAMINASE

A KINETIC STUDY ON THE INHIBITION OF THE ENZYME BY ATP

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Summary

1. Rat skeletal muscle AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) at optimal KCl concentrations shows a biphasic response to increasing levels of the allosteric inhibitor ATP.

2. Up to 10 μM, ATP appears to convert the enzyme to a form exhibiting sigmoidal kinetics while at higher concentrations its inhibitory effect is manifested by an alteration of AMP binding to AMP deaminase indicative of negative homotropic cooperativity at about 50% saturation.

3. AMP deaminase is inactivated by incubation with the periodate oxidation product of ATP. The (oxidized ATP)—AMP deaminase complex stabilized by $NaBH_4$ reduction shows kinetic properties similar to those of the native enzyme in the presence of high ATP concentrations.

4. A plausible explanation of the observed cooperativity is that ATP induces different conformational states of AMP deaminase subunits, causing the substrate to follow a sequential mechanism of binding to enzyme.

5. Binding of the radioactive oxidized ATP shows that 3.2 mol of this reagent bind per mol AMP deaminase.

Introduction

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6), which catalyzes the hydrolytic deamination of AMP to IMP and NH_3, is widely distributed in animal tissues but its level of activity in skeletal muscle is particularly high as compared with that found in all other tissues, including heart and smooth muscle [1,2]. White muscles have a higher AMP deaminase content than red muscles [3]. A recent report showed that different types of AMP deaminase exist in white and red muscles and that the higher AMP deaminase activity of white muscles is accounted for by an enzyme form which is present in much lower amount in red muscles [4].

The function of AMP deaminase in muscle operation has been unclear up to now. However, on the basis of the considerably high specific activity of the enzyme in skeletal muscle, there is no doubt that in this tissue AMP deaminase may play a significant role in regulating the relative concentration of adenine nucleotides.

The kinetic and regulatory properties of skeletal muscle AMP deaminase markedly depend on the concentration and kind of salt present in the assay mixture [5,6]. At optimal KCl concentrations (50—100 mM) the enzyme follows hyperbolic kinetics. In these conditions AMP deaminase is inhibited by GTP which induces positive cooperativity in the enzyme. A sigmoidal substrate-velocity curve similar to that obtained in the presence of GTP is shown by the enzyme at K^+ concentrations lower than optimal. In both cases ADP reverts the sigmoidal kinetic to a hyperbolic one [6,7].

The effect of ATP on skeletal muscle AMP deaminase activity is rather more complex. It has long been thought that ADP and ATP have a common effect in activating the enzyme [8]. The data of a comparative study on the regulatory properties of AMP deaminase from skeletal muscle of 5 different species showed that at the K^+ concentrations which give the complete activation of the enzyme, ATP acts as an inhibitor [5]. At K^+ concentrations lower than optimal ATP shows a biphasic regulatory effect; up to 5 μM ATP a net inhibition of AMP deaminase activity is observed, while higher ATP concentrations slightly activate the enzyme [5,6,9].

Our suggestion that in the biphasic regulation by ATP only the inhibitory effect is likely to be physiologically significant [5,6] has recently been confirmed by another research group [7]. However, it should be pointed out that although these authors claim that the effects of GTP and ATP are indistinguishable in inducing positive homotropic cooperativity in AMP deaminase, no detailed study of the nature of the inhibition by ATP has been carried out. In view of the observation that two different classes of binding sites for ATP and GTP, 3.8 and 2.0 respectively, were found in the rabbit enzyme [9], differences in the inhibitory effects of these nucleotides seem to be likely.

Our previous paper showed that treatment of rat muscle AMP deaminase with periodate-oxidized GTP specifically modifies two binding sites inducing in the enzyme a positive homotropic effect similar to that of the native enzyme in the presence of GTP [10]. In the present report, we have investigated the nature of the inhibition of AMP deaminase by ATP. Together with the effect of various ATP concentrations on the kinetic behaviour of the enzyme at optimal K^+ concentrations, we have studied the binding by AMP deaminase of the periodate oxidation product of ATP as well as the kinetic properties of the (oxidized ATP)—AMP deaminase derivative. The data obtained show that besides qualitative dissimilarities in the GTP and ATP effects on AMP deaminase activity, also differences exist in the number of binding sites for the oxidized nucleotides.

Materials and Methods

Enzyme

Rat muscle AMP deaminase was purified as previously described [10]. The

enzyme showed a specific activity of 1200 μmol AMP deaminated per min per mg protein when assayed at 20°C in 50 mM imidazole-HCl (pH 6.5), 100 mM KCl and 2 mM AMP. Protein concentration was calculated from the absorbance at 280 nm assuming an $E^{1\%}_{1cm}$ value of 9.8 [11]. The molecular weight of the enzyme was taken as 290 000 [11]. Enzyme activity was assayed spectrophotometrically [12] following the increase in optical density at 285 nm and using a ΔE_{mM} of 0.23 to calculate the amount of AMP deaminated [13].

Inactivation studies

The periodate oxidation product of ATP was prepared essentially as has been described [14]. The concentration of the oxidized ATP was calculated spectrophotometrically by using a molar extinction coefficient of 15 000 at 260 nm and pH 7.0. Incubation of the enzyme with different concentrations of periodate oxidized ATP was carried in 0.04 M succinate/0.4 M Tris-HCl (pH 8.0)/ 200 mM KCl, at 20°C. At intervals samples of the reaction mixture were removed and immediately tested for catalytic activity using a 200-fold dilution in 50 mM imidazole-HCl (pH 6.5), 100 mM KCl and 0.1 mM AMP. Control samples were incubated in the absence of oxidized ATP. To prepare a stable (oxidized ATP)-enzyme complex, the enzyme inhibited by incubation with oxidized ATP was reduced by the addition of 20 μl of a fresh aqueous solution of 1 M $NaBH_4$ to each ml of the incubation mixture. The reduction was allowed to proceed for 30 min at 0°C and the reaction mixture was then dialysed for 24 h against several changes of 0.8 M KCl/0.02 M phosphate buffer (pH 6.9) at 4°C.

Analytical measurements

The number of oxidized ATP equivalents bound to AMP deaminase was determined by treating the enzyme with the radioactive reagent obtained by periodate oxidation of [^{3}H]ATP. The incubation was carried out in 0.04 M succinate/0.4 M Tris-HCl (pH 8.0) and 200 mM KCl for about 60 min, when no further change in enzyme activity occurred. After $NaBH_4$ reduction and dialysis, the radioactivity and the specific activity of the enzyme were determined. Calculations of mol oxidized [^{3}H]ATP bound per mol enzyme were based on a specific radioactivity of oxidized [^{3}H]ATP of $12 \cdot 10^7$ cpm/μmol and a molecular weight of AMP deaminase of 290 000. For the determination of the number of binding sites, the slope and intercept of the double reciprocal plot of oxidized [^{3}H]ATP bound as a function of the concentration of free reagent were calculated by fitting the data to a straight line by the method of least squares, with a weight $\bar{v}^4$ for each point to allow for the effect of taking reciprocals [15].

Protein concentration was determined by the method of Lowry et al. [16] using native AMP deaminase as standard. Determination of radioactivity was carried out in a Packard model 2002 TriCarb liquid scintillation spectrometer in the solvent system described by Bray [17]. Spectrophotometric determinations were made in a Zeiss PM QII spectrophotometer equipped with a Zeiss TE converter and a GOERZ Servogor S recorder. At AMP levels below 0.6 mM the change in absorbance was followed on an expanded scale. AMP and ATP were purchased from Sigma Chemical Co., St. Louis, Mo.,

U.S.A.; [2-^{3}H]ATP from the Radiochemical Centre, Amersham, Bucks., U.K.; the other reagents were of analytical grade.

Results

Effect of various ATP concentrations on AMP deaminase saturation kinetics

In the experiments to study the effect of ATP concentration on rat muscle AMP deaminase activity we used as reaction mixture 50 mM imidazole-HCl (pH 6.5) and 60 mM KCl, which gives the maximal activation to the enzyme at 0.1 mM AMP [6]. Fig. 1 shows that in these conditions 2 μM ATP causes an inhibition of 70%; when the ATP concentration is raised over the range from 5 to 75 μM the inhibitory effect is partially removed, but is again restored by a further increase of ATP concentration. The rather puzzling feature of the effect of ATP on enzyme activity is related to the existence of at least two qualitatively different influences of this inhibitor in altering the affinity of the enzyme for AMP. This is shown by Fig. 2, which illustrates the effect of three different concentrations of ATP on the substrate saturation curve of rat muscle AMP deaminase. In the absence of ATP, the enzyme follows a nearly hyperbolic saturation kinetics, with a Hill coefficient of 1.2. The inhibition by 10 μM ATP causes the hyperbolic curve to revert to the sigmoidal one shown by the enzyme at KCl concentrations lower than optimal [6,7]. The corresponding Hill plot has a slope of 2.0. In the presence of 50 μM ATP, the enzyme displays a quite different substrate saturation curve. Although at AMP concentrations lower than 0.3 mM the enzyme activity in the presence of 50 μM ATP is higher than that in the presence of 10 μM ATP, betwen 0.3 and 1.0 mM AMP the inhibition caused by 50 μM ATP is more powerful. Thus, 50 μM ATP appears to induce the transition of the enzyme to a form exhibiting negative cooperativity, as is shown by the Hill plot of the data, which at intermediate substrate concentrations shows slope less than 1.0. AMP deaminase in

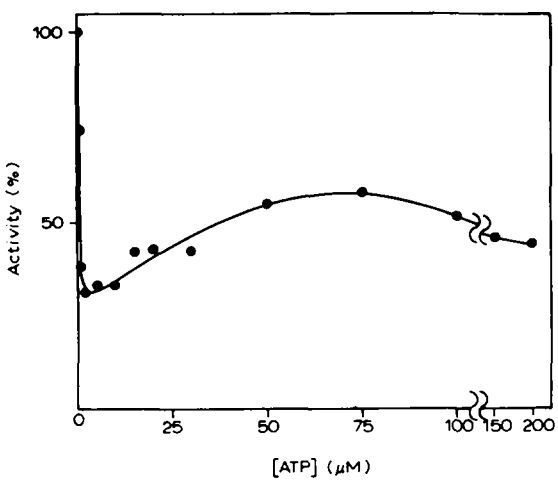

Fig. 1. Activity of rat muscle AMP deaminase as a function of ATP concentration. The reaction mixture contained 50 mM imidazole-HCl (pH 6.5), 60 mM KCl and 0.1 mM AMP.

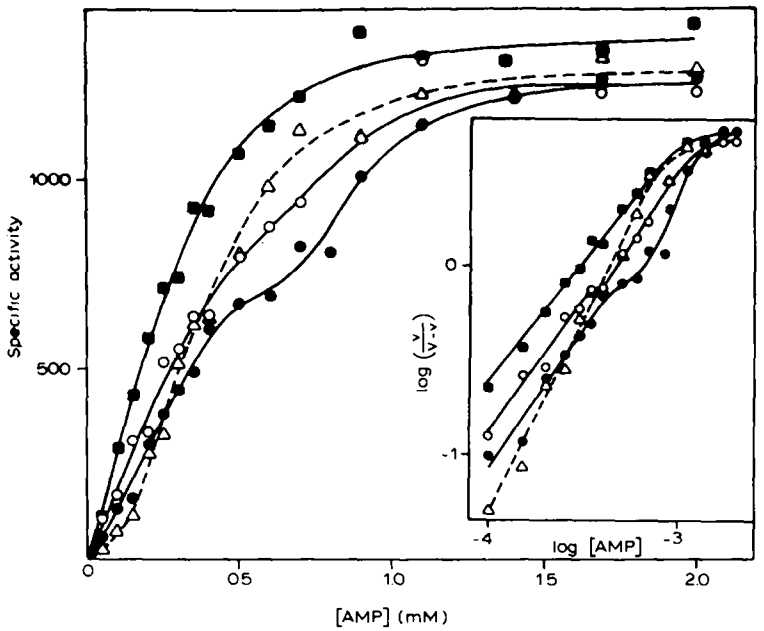

Fig. 2. Effect of ATP concentration on substrate saturation curve of AMP deaminase. Assays were performed in 60 mM KCl/50 mM imidazole-HCl (pH 6.5), with the reported AMP concentration either in the absence (■) or in the presence of ATP, 10 μM (△), 50 μM (○), and 200 μM (●). Inset, Hill plots of the substrate-velocity data.

the presence of 200 μM ATP shows a kinetic behaviour similar to that of the enzyme in the presence of 50 μM ATP with an accentuation of the inhibitory effect mainly at the middle of the saturation curve.

Inactivation of AMP deaminase by the periodate oxidation product of ATP

The incubation of rat muscle AMP deaminase with oxidized ATP prepared as described in Materials and Methods causes a rapid inactivation of the enzyme (Fig. 3). Both the rate and the extent of inactivation depend on the reagent concentration. After 60 min incubation, when the reactions are complete, inhibitions of 30, 44, 66 and 83% are observed in the presence of 3, 8, 20 and 40 μM oxidized ATP, respectively. The extent of inhibition obtained at the completion of the reaction strongly decreased when it was determined at substrate concentrations higher than K_m (0.5 mM AMP [11]). With 40 μM oxidized ATP, AMP deaminase retained 17 and 38% of the original activity when the AMP concentration used in the assay mixture were 0.1 mM and 2.0 mM, respectively (Fig. 3). This phenomenon could be interpreted as due to a substrate-induced dissociation of the enzyme-inhibitor complex. However, the (oxidized ATP)-AMP deaminase derivatives stabilized by $NaBH_4$ reduction retained the same degrees of inactivation observed before reduction at both high and low AMP concentrations. The substrate concentration effect may rather be related to the nature of the inactivation process, which is essentially due to an allosteric mechanism, as is shown by Fig. 4, which compares the double reciprocal plot of the native enzyme activity with those of the enzymes

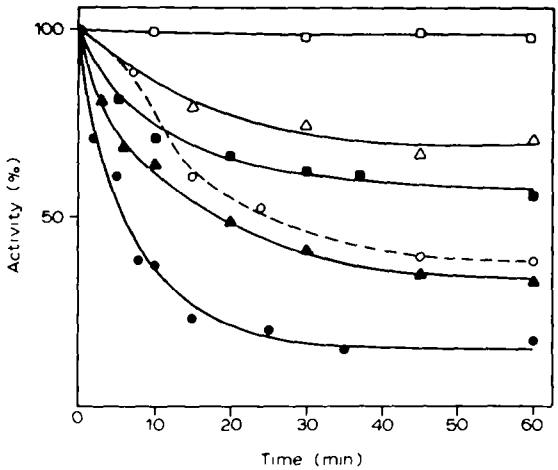

Fig. 3. Inactivation of rat muscle AMP deaminase by periodate-oxidized ATP. The enzyme, 0.7 μM, was incubated in 0.04 succinate/0.4 M Tris-HCl (pH 8.0)/200 mM KCl with oxidized ATP, 3 μM (△), or 8 μM (■), or 20 μM (▲), or 40 μM (●). The enzyme reacted with 40 μM oxidized ATP was also assayed in the presence of 2 mM AMP (○). Control samples without oxidized ATP (□).

which had been treated with 4 different concentrations of oxidized ATP. A marked decrease in V is observed only in the enzyme inactivated by 50 μM oxidized ATP, while the V values of the enzymes treated with 2—30 μM oxidized ATP do not noticeably differ from that of the native enzyme. This observation indicates that the decreased enzyme activity after slight inactivation by oxidized ATP results from alteration of AMP deaminase affinity for substrate. The double reciprocal plots of the partially inactivated enzymes do

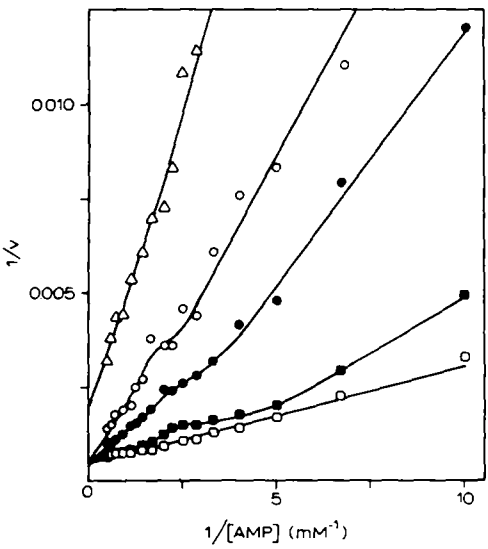

Fig. 4. Lineweaver-Burk plots of native and reduced (oxidized ATP)-AMP deaminase activities. The reaction mixture contained 50 mM imidazole-HCl (pH 6.5) and 60 mM KCl. The modified enzymes were stabilized after treatment with oxidized ATP, 2 μM (■), 4 μM (●), 30 μM (○) and 50 μM (△). □, native enzyme.

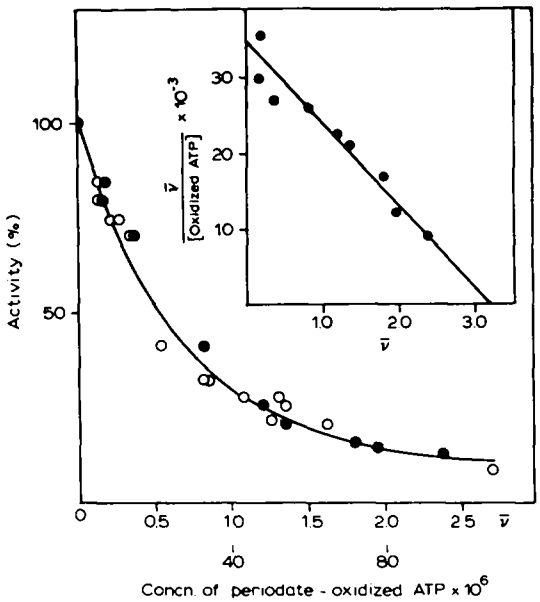

Fig. 5. Correlation between AMP deaminase inactivation and binding of oxidized ATP. ○, residual enzyme activity versus oxidized ATP concentration; ●, residual enzyme activity versus $\bar{\nu}$ (mol of oxidized [^{3}H]-ATP bound per mol of enzyme after $NaBH_4$ reduction). Inset, Scatchard plot of the binding data.

not fit with a straight line but shows a concave downward deviation which may be interpreted as being due to a negative cooperativity effect. This may be appreciated by plotting the data in the Hill form, where the Hill coefficient assumes values lower than 1.0 in the intermediate part of the curves.

Binding of the periodate oxidation product of ATP by AMP deaminase

The residual activities of AMP deaminase (0.7 μM) after 60 min incubation with the indicated concentrations of oxidized ATP are plotted in Fig. 5, which also shows the ratio ($\bar{\nu}$) of mol of periodate-oxidized [^{3}H]ATP bound per mol of enzyme at various degrees of inactivation obtained by treating the enzyme with different concentration of the radioactive reagent. All the results of Fig. 5 up to 85% loss of activity fit with an exponential decay curve, so that a straight line is obtained when they are plotted semilogarithmically. The Scatchard plot of the data (Fig. 5) as well as the double reciprocal plot of $\bar{\nu}$ as a function of the concentration of free reagent were linear. The number of binding sites determined corresponded to 3.20 ± 0.10 mol oxidized ATP/290 000 g protein, with an apparent dissociation constant of 86 ± 6,6 μM.

Discussion

The substrate-velocity curve of skeletal muscle AMP deaminase at KCl concentrations lower than 50 mM is sigmoid shaped [6,7,18]. This kinetic behaviour is observed also in the enzyme at optimal monovalent cation concentration (50—100 mM KCl) in the presence of GTP [7,9,13] or in the presence of 10 μM ATP, as shown in this paper. Positive cooperativity in skeletal muscle

AMP deaminase is unlikely to be physiologically significant. First, in the absence of effectors this phenomenon is observed in AMP deaminase at KCl concentrations considerably lower than those (100—150 mM) found in mucle. Secondly, in AMP deaminase at optimal salt concentrations, this kinetic behaviour is induced by ATP and GTP over a concentration range from 10^{-7} to 10^{-5} M. However, when the ATP concentration is increased to values closer to those (10^{-3} M) found in muscle [19] the inhibitory effect as well as the binding of GTP is abolished [5,9] and, as shown in this paper, the enzyme displays a quite different kinetic behaviour. The shape of the Hill plots based on substrate saturation kinetics in the presence of 50—200 μM ATP shows a deviation from linearity in the region of about 50% saturation. A comparison with the theoretical plots calculated by inserting in the Hill function plausible values of four consecutive binding ratios [20] indicates a similarity of our experimental curves with that representing the case of a mixture of negative and positive cooperativity. Since skeletal muscle AMP deaminase exhibits kinetics markedly dependent on protein concentration [21] as well as on the kind and concentration of cations and anions present in the assay mixture [6], the possible interpretations of the effect of each modifier on AMP deaminase kinetics will be resolved only by reduction of the number of the variables affecting the enzyme activity. For this purpose, a useful approach can be the specific modification of binding sites for the effectors, as recently shown with the treatment of rat muscle AMP deaminase with pyridoxal 5′-phosphate and periodate-oxidized GTP [10,22]. The results presented in this paper show that the treatment with periodate-oxidized ATP of AMP deaminase produces a decrease of enzymic activity which is made irreversible by reduction with $NaBH_4$. The (oxidized ATP)-AMP deaminase complex behaves similarly to the native enzyme in the presence of high ATP concentrations in showing negative homotropic cooperativity even at optimal KCl concentration. Since the treatment with oxidized ATP was carried out at protein and salt concentrations where skeletal muscle AMP deaminase behaves as a tetramer [23], we may assume that the alteration of the enzyme kinetics observed in these conditions results from a conformational change which is not sufficient to destroy the subunit attractions, but causes AMP to follow a sequential mechanism of binding to the enzyme.

Binding of radioactive oxidized ATP fits with a hyperbolic saturation curve, indicating binding of 3.20 ± 0.1 mol oxidized ATP per mol protein with an apparent dissociation constant of $86 \cdot 10^{-6}$ M. These results are in agreement with those of Tomozawa and Wolfenden [9] who found 3.8 binding sites for ATP with an apparent dissociation constant of $21 \cdot 10^{-6}$ M by equilibrium binding studies with rabbit skeletal muscle AMP deaminase. From these data we can deduce that the qualitatively different effects of periodate-oxidized ATP and GTP in altering AMP deaminase kinetics can be related to marked quantitative differences in the binding of these reagents to the enzyme, since only 1.9 binding sites for oxidized GTP, with an apparent dissociation constant of $6.8 \cdot 10^{-6}$ M were found in rat muscle AMP deaminase [10].

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References

1 Conway, E.J. and Cooke, R. (1939) Biochem. J. 33, 479—492
2 Purzycka, J. (1962) Acta Biochim. Polon. 9, 83—93
3 Raggi, A., Ronca-Testoni, S. and Ronca, G. (1969) Biochim. Biophys. Acta 178, 619—622
4 Raggi, A., Bergamini, C. and Ronca, G. (1975) FEBS Lett. 58, 19—23
5 Ronca-Testoni, S., Raggi, A. and Ronca, G. (1970) Biochim. Biophys. Acta 198, 101—112
6 Ronca, G., Raggi, A. and Ronca-Testoni, S. (1972) Ital. J. Biochem. 21, 305—321
7 Coffee, C.J. and Solano, C. (1977) J. Biol. Chem. 252, 1606—1612
8 Zielke, C.L. and Suelter, C.H. (1971) Enzymes 4, 47—77
9 Tomozawa, Y. and Wolfenden, R. (1970) Biochemistry 9, 3400—3404
10 Ranieri-Raggi, M. and Raggi, A. (1976) Biochim. Biophys. Acta 445, 223—233
11 Ronca-Testoni, S., Ranieri, M., Raggi, A. and Ronca, G. (1970) Ital. J. Biochem. 19, 262—276
12 Kalckar, H.M. (1947) J. Biol. Chem. 167, 461—475
13 Ronca, G., Raggi, A. and Ronca-Testoni, S. (1968) Biochim. Biophys. Acta 167, 626—629
14 Lamed, R., Levin, Y. and Wilchek, M. (1973) Biochim. Biophys. Acta 304, 231—235
15 Cornish-Bowden, A.J. (1976) Principles of Enzyme Kinetics, pp. 173—184, Butterworths, London
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Bray, G.A. (1960) Anal. Biochem. 1, 279—285
18 Smiley, K.L., Jr. and Suelter, C.H. (1967) J. Biol. Chem. 242, 1980—1981
19 Seraydarian, K., Mommaerts, W.F.H.M. and Wallner, A. (1962) Biochim. Biophys. Acta 65, 443—460
20 Cornish-Bowden, A. and Koshland, D.E. Jr. (1975) J. Mol. Biol. 95, 201—212
21 Hemphill, R.M., Zielke, C.L. and Suelter, C.H. (1971) J. Biol. Chem. 246, 7237—7240
22 Ronca-Testoni, S. and Ronca, G. (1974) J. Biol. Chem. 249, 7723—7728
23 Ashman, L.K. and Atwell, J.L. (1972) Biochim. Biophys. Acta 258, 618—625