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INTERACTION OF PIG KIDNEY AND LENTIL SEEDLING COPPER-CONTAINING AMINE OXIDASES WITH GUANIDINIUM COMPOUNDS

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The effect of guanidinium compounds on the catalytic mechanism of pig kidney and lentil seedling amine oxidases has been investigated by polarographic techniques and spectroscopy. Guanidine does not inhibit the lentil enzyme and is a weak inhibitor for pig kidney amine oxidase ($K_i = 1$ mM), whereas aminoguanidine is an irreversible inhibitor of both enzymes, with a K_i value of 10^{-6} M. 1,4-Diguanidino butane (arcaine) is a competitive inhibitor for both pig and lentil amine oxidases. Amiloride is a competitive inhibitor for pig enzyme, but upon prolonged incubation with this drug the enzyme gradually loses its activity in an irreversible manner.

Keywords: Pig kidney; *Lens esculenta*; Amine oxidase; Copper; 6-Hydroxydopa; Guanidine; Arcaine; Amiloride

Abbreviations: Amiloride, 1-(3,5-diamino-6-chloropyrazinecarboxyl)guanidine; AOs, amine oxidases; Arcaine, 1,4-diguanidino butane; LSAO, lentil seedling amine oxidase; PKAO, pig kidney amine oxidase; TPQ, 6-hydroxydopa quinone (2,4,5-trihydroxyphenylalanine)

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INTRODUCTION

Amine oxidases (AOs) (amine oxygen oxidoreductase deaminating, copper containing; E.C. 1.4.3.6) catalyse the oxidative deamination of primary amines, including mono-, di- and polyamines, to the corresponding aldehydes, ammonia and hydrogen peroxide. AO activity is found in a wide range of bacteria, fungi, plants and animals.[1] Well studied examples in mammals are AOs from pig kidney,[2] human placenta,[3,4] bovine serum[5] and equine plasma,[6] while in plants the best known and studied are the enzymes of *Lens* and *Pisum* seedlings.[7] AOs are soluble dimeric enzymes containing two copper atoms and two organic prosthetic groups identified as TPQ, the diquinone of a 2,4,5-trihydroxyphenylalanyl residue, in the bovine serum enzyme.[8–10] Subsequently TPQ has been identified in many copper-containing AOs.[5] AOs from *Hansenula polymorpha*,[11] lentil[12] and pea seedlings[13] have been cloned and sequenced. The sequence suggested that TPQ is formed by post-translational modification of a tyrosine residue in the active site and that this residue is conserved in all copper-containing AOs.[14]

The study of enzyme inhibition by substrate analogues is a useful tool in probing structure–function relationships in enzyme action. Recently amiloride and some of its derivatives have been used to inhibit several Na^+ transporting systems,[15] and it has been demonstrated that human placental AO was the previously described amiloride-binding protein. This enzyme like pig kidney and bovine serum AO was inhibited by amiloride analogues.[16–18] Several inhibitors of plant copper AOs have been described[19] and recently a competitive inhibition of lentil AO by amiloride and other compounds has been reported.[20]

Since amiloride has a guanidinium function, and guanidines have been reported to be good inhibitors of AOs[21] this paper reports on the interactions of guanidinium compounds with pig kidney AO (PKAO) compared with lentil seedling AO (LSAO).

EXPERIMENTAL PROCEDURES

Guanidine hydrochloride (Figure 1(a)), aminoguanidine hydrochloride (Figure 1(b)), agmatine sulfate, putrescine hydrochloride, arcaine sulfate (Figure 1(c)), amiloride hydrochloride (Figure 1(d)), and beef liver catalase were from Sigma Chemical Company (USA), and used without further purification. AO was purified from lentil (*Lens esculenta*) seedlings (LSAO) as previously described.[22] An ε_{498} of $4100\,M^{-1}cm^{-1}$ or an ε_{278} of

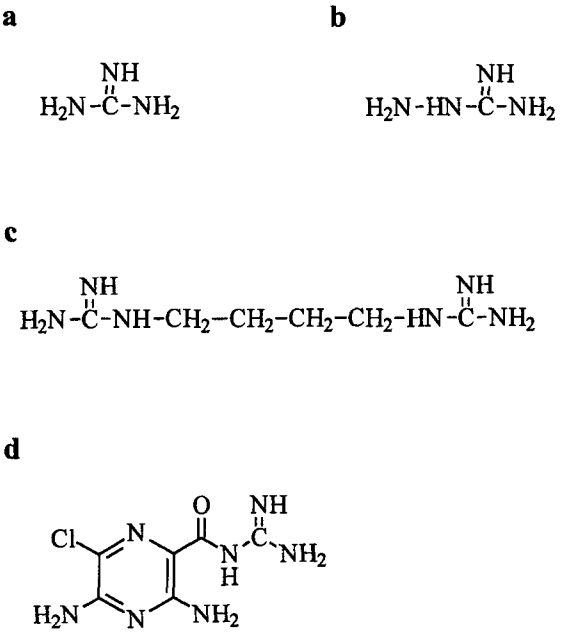

FIGURE 1 Chemical structures of guanidine (a), aminoguanidine (b), arcaine (c) and amiloride (d).

$2.45 \times 10^5\,M^{-1}\,cm^{-1}$ for the purified enzyme (two copper ions and a M_r of 150 000) was used to estimate enzyme concentration.[23] Pig kidney enzyme (PKAO) was purified according to the procedure described.[24] The final preparation showed the characteristic absorption spectrum of copper-containing AOs (A_{278}/A_{490} nm = 75). An ε_{490} of $4000\,M^{-1}\,cm^{-1}$ or an ε_{278} of $3 \times 10^5\,M^{-1}\,cm^{-1}$ for the purified enzyme (two copper ions and a molecular mass of 180 kDa) was used to estimate enzyme concentration. For both enzymes, one unit of enzyme activity corresponded to 1 μmol substrate oxidised per minute. Absorption spectra were recorded at room temperature with a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Anaerobic experiments were made after several cycles of evacuation followed by flushing with O_2-free Argon at 25°C in a Thunberg-type spectrophotometer cuvette, where anaerobic additions of various reagents could be made through a rubber cap with a syringe. Oxygen uptake was determined polarographically by a Gilson Oxygraph equipped with a Clark electrode. The standard reaction mixture (1 ml) contained the enzyme (5×10^{-3} μM LSAO; 250×10^{-3} μM PKAO) in 100 mM KP_i buffer, pH 7, and 37°C. The reaction was started by addition of substrate (between 5×10^{-5} and 2×10^{-4} M) after

at least 10 min preincubation. Each inhibitor was tested in the presence and absence of 100 μg crystalline beef liver catalase. Under these conditions the measure of AO activity is fairly reproducible and linear as a function of enzyme concentration. Inhibition constants were determined by Dixon plots.[25] The constant k_2 and the apparent inhibition constant K_i were obtained from double reciprocal plot of the inactivation rate constants ($1/K_{app}$) versus $1/[I]$ from the intercept on the x-axis and y-axis respectively.

RESULTS AND DISCUSSION

Reaction of AOs with Agmatine

Agmatine was a good substrate for PKAO. In 100 mM KP_i buffer, pH 7, the K_m value was similar to that for the best substrate cadaverine (0.1 mM), and the turnover number was found to be 84 min^{-1}, about 40% of the corresponding number (210 min^{-1}) found for cadaverine. Agmatine was a substrate also for LSAO with a K_m value similar to that of putrescine (0.1 mM), but the turnover number in 100 mM KP_i buffer, pH 7, was 1400 min^{-1}, i.e. only 15% of the corresponding number (9300 min^{-1}) found for putrescine (results not shown).

Reaction of AOs with Guanidine and Aminoguanidine

Guanidine did not inhibit LSAO, but is a noncompetitive inhibitor of PKAO with a K_i of 1 mM. Aminoguanidine was instead an irreversible inhibitor for both enzymes, with a K_i of 1 μM for PKAO and 5 μM for LSAO (not shown).

Reaction of AOs with Arcaine

Arcaine has been found to be a competitive inhibitor for pig and horse kidney AOs.[26,27] In the case of PKAO arcaine has a $K_i = 20\,\mu M$. Arcaine was shown to be a competitive inhibitor also for LSAO, with a $K_i = 10$ mM (not shown).

Reaction of AOs with Amiloride

The above reported experiments induced us to study the interaction between amiloride and AOs. Amiloride and its derivatives phenamyl- and

ethylpropyl- are shown to inhibit PKAO, previously found to be identical to the amiloride-binding protein.[16–18] Amiloride was confirmed to behave as a competitive inhibitor of PKAO ($K_i = 10^{-4}$ M). However after prolonged incubation it irreversibly inhibited the enzyme. The time-dependence for irreversible inhibition was checked by preincubating PKAO at 37°C in the presence of three concentrations of amiloride (Figure 2). The rate of loss of enzyme activity increased linearly with increased amiloride concentration, the inactivation apparently following first order kinetics. A double reciprocal plot of the inactivation rate constants against the amiloride concentration yielded a limiting inactivation rate constant (k_2) of 0.13 min^{-1} and an apparent inhibition constant K_i of 0.9 mM (Figure 3). This result is consistent with the reversible formation of an enzyme–inhibitor complex [E · I], followed by an irreversible inactivation due to the generation of a stable enzyme–inhibitor complex [E–I]:

$$E + I \underset{k_{-1}}{\overset{k_1}{\Longleftrightarrow}} [E \cdot I] \xrightarrow{k_2} E\text{–}I \qquad (1)$$

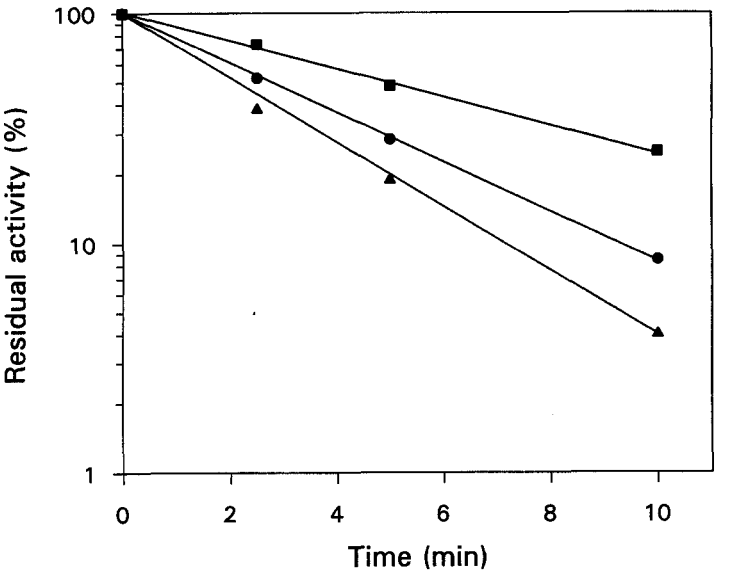

FIGURE 2 Inactivation of PKAO by amiloride. The enzyme (0.32 μM) was preincubated with the indicated concentrations of amiloride at 37°C in 100 mM KP_i, pH 7. Concentrations of amiloride were: (■) 5 μM; (●) 7 μM; (▲) 15 μM.

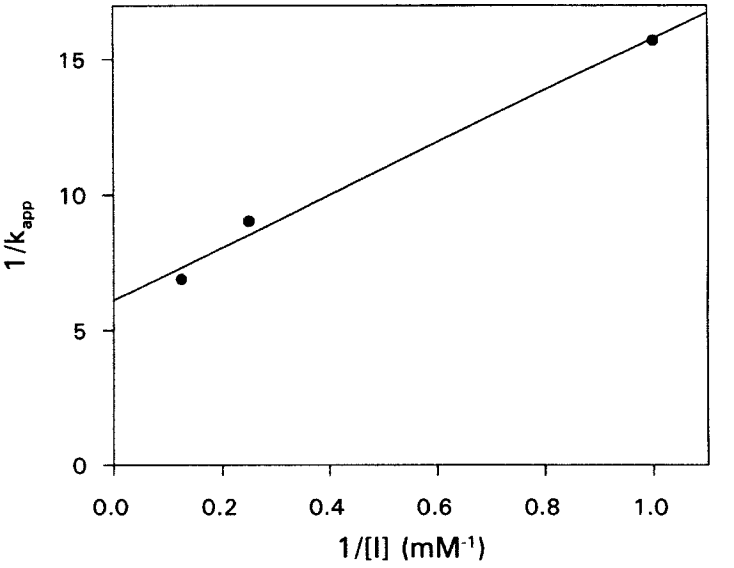

FIGURE 3 Double reciprocal plot of apparent first order rate constant (k_{app}) versus amiloride concentrations. The data obtained were extrapolated from the data in Figure 2.

No detectable inactivation was obtained using dimethyl sulfoxide, the solvent for the amiloride reagent. The irreversibility of the reaction between PKAO and amiloride was confirmed by the lack of reactivation of the enzyme even after exhaustive dialysis against buffer.

In contrast amiloride reacts in a competitive reversible way with LSAO ($K_i = 10^{-3}$ M).

Spectrophotometric Features

In addition to absorption by amino acids in the UV region, PKAO absorbs specifically in the visible region (490 nm), owing to the TPQ cofactor. Reaction with a substrate causes the immediate disappearance of the 490 nm absorption band indicating the rapid formation of a reduced enzyme. The addition of arcaine to PKAO did not affect the 490 nm absorption but a new peak at 390 nm becomes apparent both in anaerobic and in air-satured samples (Figure 4). Since arcaine does not absorb at 390 nm, this peak must be due to the formation of an enzyme–inhibitor complex. This complex is reversible as demonstrated by the fact that the enzyme regains its native absorption spectrum and activity after dialysis.

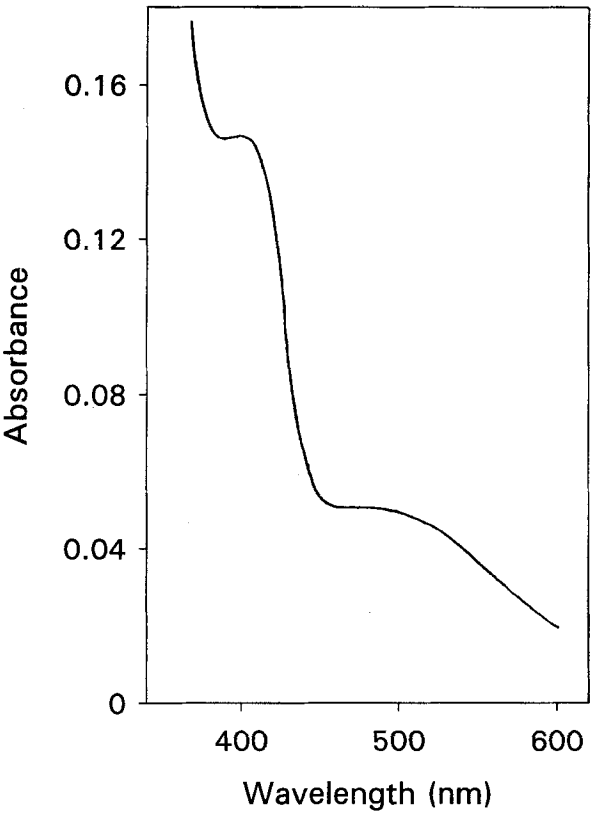

FIGURE 4 Absorption spectrum of 12.5 μM PKAO after 10 min reaction with 2 mM arcaine in 100 mM KP_i buffer, pH 7, under anaerobic conditions.

It is worth recalling that phenylhydrazine irreversibly inactives copper AOs.[28] Titration of PKAO with phenylhydrazine yields an intense absorption peak at 442 nm, with a stoichiometry of 1.9 ± 0.2 phenylhydrazine per dimer, similar to that found for LSAO.[23] The PKAO–arcaine complex still reacted with phenylhydrazine. In fact the addition of phenylhydrazine to arcaine-treated PKAO gave rise to the typical 442 nm absorption band with concomitant decrease of the 390 nm absorption. The final stoichiometry for the reaction was 2 mol phenylhydrazine per mol PKAO as in the native enzyme. However while in the latter case the reaction was over in less than 2 min, about 1 h was required in the case of arcaine-treated PKAO. These results indicate that arcaine binds the TPQ cofactor with the formation of a reversible enzyme–inhibitor complex.

When the same experiment was conducted with LSAO, a different pattern arose. The incubation of LSAO with arcaine did not form the 390 nm absorption band, and the addition of phenylhydrazine was over in less than 2 min, as in the native enzyme.

The band at 390 nm was also formed by incubating PKAO with amiloride both in the presence and absence of air, and this band did not disappear after prolonged dialysis (Figure 5). The addition of phenylhydrazine to the amiloride-inactivated PKAO gave only about 10–15% of the absorption at 442 nm after 24 h incubation. This indicates the formation of a very stable complex between PKAO and amiloride.

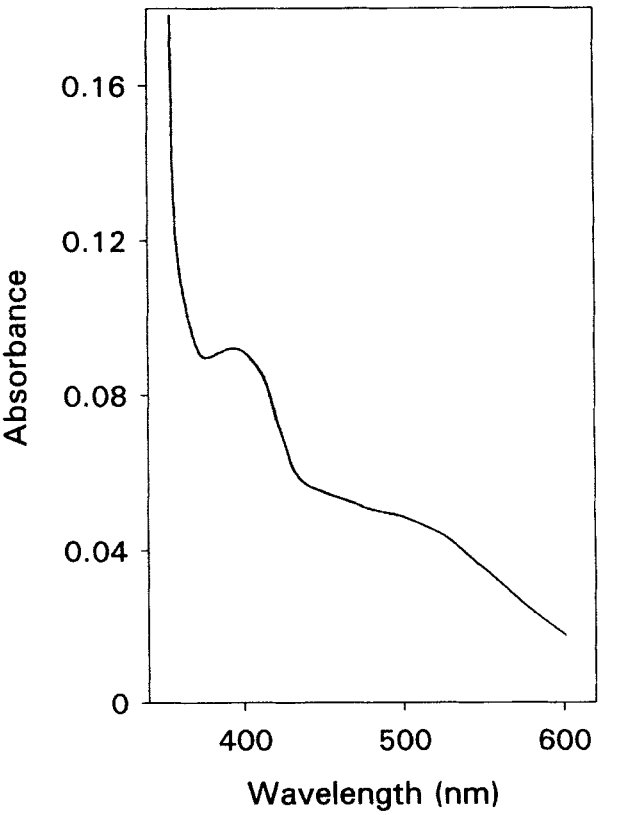

FIGURE 5 Absorption spectrum of 12.5 μM PKAO after reaction with 2 mM amiloride in 100 mM KP_i buffer, pH 7, under aerobic conditions. The spectrum was recorded after 3 h incubation and through dialysis in 100 mM KP_i buffer, pH 7.0.

CONCLUSION

The results of the present study clearly show some differences between lentil AO and PKAO.

(i) Substrate specificity. LSAO catalyses very efficiently the oxidative deamination of diamines such as putrescine and cadaverine, whereas agmatine was oxidised at slower rate (15%) with respect to the best substrate putrescine. PKAO exerts its physiological function by acting on the naturally occurring aliphatic diamines and histamine, and agmatine was oxidised at about 40% the rate of the best substrate cadaverine.

(ii) Guanidine was not an inhibitor for lentil enzyme, while it is a non-competitive inhibitor for PKAO. Aminoguanidine was an irreversible inhibitor for both enzymes.

(iii) Arcaine reacts with PKAO to form a reversible complex, as detected by the formation of a new absorption band at 390 nm, while the 490 nm absorption was unaffected. The enzyme treated with arcaine retained its ability to combine with phenylhydrazine. On the contrary, arcaine did not show any new absorption band with LSAO.

(iv) Amiloride was shown to be a competitive inhibitor for mammalian AOs.[16–18] Upon prolonged incubation with amiloride PKAO gradually loses activity in an irreversible manner. LSAO instead reversibly binds amiloride.

These data confirm that although the overall mechanism of AO action is very similar if not even identical in the two enzymes, the binding site for substrate shows significant differences which most probably are reflected by different physiological roles. It is at present not known whether the complex PKAO–amiloride will be intrinsically stable, but guanidinium compounds are very good reagents to investigate the structure of the active site of copper AOs.

Acknowledgements

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References

[1] W. McIntire and C. Hartmann (1992) In *Principles and Applications of Quinoproteins*, (V.L. Davidson, Ed.), Chap. 6. Marcel Dekker, Inc.; New York.

[2] V. Steinebach, B.W. Groen, S.S. Wijmenga, W.M.A. Niessen, J.A. Jongejan and J.A. Duine (1995) *Anal. Biochem.*, **230**, 159–166.
[3] M.J. Crabbe and W.G. Bardsley (1974) *Biochem. J.*, **139**, 183–189.
[4] F. Morel, A. Surla and P.V. Vignais (1992) *Biochem. Biophys. Res. Comm.*, **187**, 178–186.
[5] S.M. Janes and J.P. Klinman (1995) *Methods Enzymol.*, **258**, 20–34.
[6] S.R. Carter, M.A. McGuirl, D.E. Brown and D.M. Dooley (1994) *J. Inorg. Biochem.*, **56**, 127–141.
[7] R. Medda, A. Padiglia and G. Floris (1995) *Phytochemistry*, **39**, 1–9.
[8] S.M. Janes, D. Mu, D. Wemmer, A.J. Smith, S. Kaur, D. Maltby, A.L. Burlingame and J.P. Klinman (1990) *Science*, **248**, 981–987.
[9] S.M. Janes and J.P. Klinman (1991) *Biochemistry*, **30**, 4599–4605.
[10] L. Morpurgo, E. Agostinelli, B. Mondovì, L. Avigliano, R. Silvestri, G. Stefanich and M. Artico (1991) *Biochemistry*, **31**, 2615–2621.
[11] P.G. Bruinenberg, M. Evers, H.R. Waterham, J. Kuipers, A.C. Arnberg and G. Ab (1989) *Biochim. Biophys. Acta*, **1008**, 157–167.
[12] A. Rossi, M. Petruzzelli and A. Finazzi Agrò (1992) *FEBS Lett.*, **301**, 253–257.
[13] A.J. Tipping and M.J. McPherson (1995) *J. Biol. Chem.*, **270**, 16939–16946.
[14] I. Frébort and O. Adachi (1995) *J. Ferment. Bioeng.*, **80**, 625–632.
[15] C. Frelin, P. Vigne, P. Barbry and M. Lazdunski (1987) *Kidney Int.*, **32**, 785–792.
[16] W.F. Novotny, O. Chassande, M. Baker, M. Lazdunski and P. Barbry (1994) *J. Biol. Chem.*, **269**, 9921–9925.
[17] R. Federico, R. Angelini, L. Ercolini, G. Venturini, A. Mattevi and P. Ascenzi (1997) *Biochem. Biophys. Res. Comm.*, **240**, 150–152.
[18] D. Mu, K.F. Medzihradszky, G.W. Adams, P. Mayer, W.M. Hines, A.L. Burlingame, A.J. Smith, D. Cai and J.P. Klinman (1994) *J. Biol. Chem.*, **269**, 9926–9932.
[19] A. Padiglia, R. Medda, J.Z. Pedersen, A. Lorrai, P. Peč, I. Frébort and G. Floris (1998) *J. Enz. Inhib.*, **13**, 311–325.
[20] L. Ercolini, R. Angelini, R. Federico, G. Rea, G. Venturini and P. Ascenzi (1998) *J. Enz. Inhib.*, **13**, 465–471.
[21] W.G. Bardsley (1985) In *Structure and Function of Amine Oxidase*, (B. Mondovì, Ed.), pp. 136–152. CRC Press Inc.; Boca Raton, Florida.
[22] G. Floris, A. Giartosio and A. Rinaldi (1983) *Phytochemistry*, **22**, 1871–1874.
[23] A. Padiglia, R. Medda and G. Floris (1992) *Biochem. Inter.*, **28**, 1097–1107.
[24] A. Rinaldi, P. Vecchini and G. Floris (1982) *Prep. Biochem.*, **12**, 11–28.
[25] M. Dixon (1953) *Biochem. J.*, **55**, 170–171.
[26] G. Floris, A. Rinaldi and A. Finazzi Agrò (1981) *It. J. Biochem.*, **30**, 250–258.
[27] M. Corda, M. Pellegrini and A. Rinaldi (1983) *It. J. Biochem.*, **33**, 304–310.
[28] A. Rinaldi, G. Floris, S. Sabatini, A. Finazzi Agrò, A. Giartosio, G. Rotilio and B. Mondovì (1983) *Biochem. Biophys. Res. Comm.*, **115**, 841–848.