Kinetic mechanism of pulmonary carbonyl reductase

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The kinetic mechanism of guinea-pig lung carbonyl reductase was studied at pH 7 in the forward reaction with five carbonyl substrates and NAD(P)H and in the reverse reaction with propan-2-ol and NAD(P)⁺. In each case the enzyme mechanism was sequential, and product-inhibition studies were consistent with a di-iso ordered bi bi mechanism, in which NAD(P)H binds to the enzyme first and NAD(P)⁺ leaves last and the binding of cofactor induces isomerization. The kinetic and binding studies of the cofactors and several inhibitors such as pyrazole, benzoic acid, Cibacron Blue and benzamide indicate that the cofactor and Cibacron Blue bind to the free enzyme whereas the other inhibitors bind to the binary and/or ternary complexes.

INTRODUCTION

Lung of guinea pig [1] and mouse [2] has been shown to contain a tetrameric carbonyl reductase (EC 1.1.1.184) that is distinct from monomeric and dimeric enzymes from other animal and human tissues [3–8], especially in its very low substrate specificity for aromatic and aliphatic carbonyl compounds and dual cofactor specificity for NADPH and NADH. The low substrate specificity and specific occurrence in lung of the enzyme have suggested a role for the enzyme in pulmonary drug metabolism. Since, of the substrates reported with the enzyme [1,2], acetone is a natural substrate with relatively high affinity under conditions near to the pH optimum of 6.0, another role in acetone metabolism has also been proposed for the enzyme. However, a more detailed kinetic study of the enzyme under physiological pH conditions would be necessary to explore further the roles of the enzyme in detoxication and acetone metabolism.

There have been only two studies on the steady-state kinetic mechanism of carbonyl reductase, and those have yielded somewhat conflicting results, with an ordered mechanism for human brain monomeric enzyme [9] and with a di-iso ordered mechanism for dog liver dimeric enzyme [8]. Since the pulmonary enzyme differs from the hepatic enzymes not only in structural properties but also in cofactor specificity, the steady-state kinetic mechanism of the guinea-pig lung enzyme was studied in detail with acetone and several xenobiotic carbonyl compounds as substrate, and with NADPH and NADH as cofactor. In addition, the mechanism of inhibition by several lung carbonyl reductase inhibitors is reported.

EXPERIMENTAL

Materials

Nicotinamide nucleotides were obtained from Oriental Yeast Co. (Tokyo, Japan), acetaldehyde was from Merck, and acetol (1-hydroxyacetone) was from Aldrich Chemical Co. α -Methyl-4-nitrobenzyl alcohol was synthesized from 4-nitroacetophenone as described

previously [10]. Cibacron Blue 3GA was purified by paper chromatography as described previously [11]. 4-Nitroacetophenone was recrystallized from ethanol, and acetone and propan-2-ol were purified by redistillation. Other chemicals were used without further purification.

Enzyme purification

Carbonyl reductase was purified from guinea-pig lung as described previously [2]; the homogeneous preparations showed specific activities of 10.0–10.5 units/mg of protein when analysed for activity with 4-nitroacetophenone as a substrate and NADPH as a cofactor at pH 6.0 [1] and for protein with bovine serum albumin as the standard [12].

Enzyme assay

Carbonyl reductase activity was determined spectrophotometrically by monitoring NAD(P)H oxidation at 340 nm, except that the oxidation rates of NAD(P)H were measured fluorimetrically at 455 nm (excitation at 340 nm) when low NADPH concentrations of less than 10 μ M were used. The reaction mixture in a volume of 2.5 ml contained 80 mM-potassium phosphate buffer, pH 7.0, NAD(P)H, the appropriate carbonyl substrate and enzyme. The reverse reaction rate was also determined fluorimetrically by recording NAD(P)H formation in 80 mM-potassium phosphate buffer, pH 7.0, containing NAD(P)⁺, the appropriate alcohol substrate and enzyme. The concentrations of cofactor, substrate and inhibitor are given in the text describing the specific experiments. Controls contained all reactants except substrate. The reaction was initiated by the addition of enzyme.

The concentrations of NAD(P)⁺ and NAD(P)H were calculated from the absorbances at 260 nm and 340 nm respectively. The acetaldehyde concentration in stock solution was determined by measuring the enzymic oxidation of NADH with yeast alcohol dehydrogenase in the presence of excess NADH at pH 6.0. The preparation of cofactors and substrate and all assays contributing data to one experimental set were performed on the same day.

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One unit of enzyme activity was defined as that amount catalysing oxidation or formation of 1 μmol of NAD(P)H/min at 25°C.

Data analysis

Initial velocities in the asymptotic region that follows Michaelis kinetics were fitted to eqn. (1) [13]:

$$v = \frac{V[A][B]}{[A][B] + K_m^A[B] + K_m^B[A] + K_i^A K_m^B} \quad (1)$$

by using a computer program for least-squares linear regression, and results are presented as double-reciprocal forms. Kinetic studies in the presence of inhibitors were carried out in an identical manner.

Equilibrium constant

The constant $K = [\text{propan-2-ol}][\text{NAD(P)}^+]/[\text{acetone}][\text{NAD(P)H}]$ was determined by the addition of enzyme to a mixture of all four reactants, in which the concentrations of cofactors and acetone were held at their respective K_m values and the concentration of propan-2-ol was varied until a system showed no change in fluorescence at 455 nm upon incubation in 80 mM-potassium phosphate buffer, pH 7.0.

RESULTS AND DISCUSSION

Initial-velocity measurements

The double-reciprocal plots of initial rate versus concentration of the varied substrate, acetone, at a fixed concentration of NADPH, yielded a series of intersecting lines (Fig. 1b). Similar patterns of initial velocity were observed when the varied substrate was NADPH (Fig. 1a). Initial-velocity studies were also performed with alternative substrates such as pyridine-4-aldehyde, 4-nitroacetophenone, acetaldehyde and acetol or NADH, and reciprocal plots of $1/v$ against $1/[\text{substrate}]$ or $1/[\text{NAD(P)H}]$ all gave families of straight lines that converge at a point to the left of the ordinate. The slopes and intercepts for each experiment were replotted against the reciprocal of the fixed substrate concentration and were linear in each case. These results are consistent with a reaction mechanism that proceeds in a sequential manner.

Table 1 compares the kinetic constants for acetone, alternative substrates and cofactors. Whereas the values of K_m^A and K_i^A were not significantly different for all the carbonyl compounds when NADPH was used as a cofactor, that of K_m^B varied widely. The Michaelis constants for pyridine-4-aldehyde and 4-nitroacetophenone were lower than those for the substrate without an aromatic ring. The result is compatible with the previous report that carbonyl reductase shows higher affinity for aromatic carbonyl compounds than for aliphatic ones [1], and suggests that hydrophobic interaction between substrate and enzyme is important in the formation of a ternary complex with the best configuration for catalysis. However, the maximum velocity for acetone was as high as those for the aromatic substrates, and the NADH/acetone combination gave the highest value of 14.7 units/mg, which is equivalent to a turnover number of 1260 min^{-1} assuming an M_r value of 86000. Acetone has been suggested to be converted into glucose through a methylglyoxal pathway or a propan-1,2-diol pathway [14,15]. The Michaelis con-

Fig. 1. Initial-velocity patterns of the reduction of acetone by guinea-pig lung carbonyl reductase

(a) Reciprocal plots with NADPH as the varied substrate. Acetone concentrations were 27 μM ($\square$), 52 μM ($\bullet$), 98 μM ($\triangle$), 208 μM ($\blacksquare$) and 415 μM ($\circ$). (b) Reciprocal plots with acetone as the varied substrate. NADPH concentrations were 1.3 μM ($\square$), 1.9 μM ($\bullet$), 2.5 μM ($\triangle$), 3.7 μM ($\blacksquare$) and 7.6 μM ($\circ$). Replots of the slopes ($\circ$) and intercepts ($\bullet$) are shown in the insets. Initial velocity (v) is expressed as units/ml.

stant of the present enzyme for acetone is much lower than the acetone concentration (0.4–9 mM) in plasma reported under starvation and conditions of ketoacidosis [16,17], and is also lower than the apparent K_m value of acetone mono-oxygenase [18] that catalyses the hydroxylation of acetone to acetol, the first step in acetone metabolism. Carbonyl reductase also reduced acetol, which suggests the enzyme as a candidate for the proposed extrahepatic enzyme that produces propan-1,2-diol as an intermediate in one pathway of acetone metabolism [14], but the affinity and velocity for acetol of the enzyme were lower than those for acetone. Therefore carbonyl reductase may be responsible for an alternative metabolic pathway of acetone to form propan-2-ol, although it is unknown whether the product is excreted unchanged or further metabolized.

When the reduced products from these carbonyl compounds were tested as substrate in the reverse

Table 1. Kinetic constants of guinea-pig lung carbonyl reductase

The constants are those of eqn. (1), where A and B are cofactor and carbonyl or alcohol substrate respectively. The values are expressed as means $\pm$ s.e.m. for three to five determinations. The ranges of concentration of cofactor and substrate were $0.7\text{--}15 \times K_m$ for NADPH, $0.8\text{--}19 \times K_m$ for NADP⁺, $0.6\text{--}3 \times K_m$ for NADH, $1\text{--}10 \times K_m$ for NAD⁺, $0.6\text{--}20 \times K_m$ for 4-nitroacetophenone, $0.5\text{--}6 \times K_m$ for pyridine-4-aldehyde, $0.25\text{--}4 \times K_m$ for acetaldehyde, $0.3\text{--}4 \times K_m$ for acetol and $0.5\text{--}20 \times K_m$ for propan-2-ol.

Cofactor/substrate	V (units/mg)	K_m^A (μM)	K_m^B (μM)	K_i^A (μM)
NADPH/acetone	6.8 ± 0.6	0.4 ± 0.1	45 ± 4	0.9 ± 0.2
NADH/acetone	14.7 ± 1.6	53 ± 10	342 ± 47	1040 ± 190
NADPH/4-nitroacetophenone	6.7 ± 0.8	1.0 ± 0.3	7.1 ± 1.2	1.6 ± 0.4
NADPH/pyridine-4-aldehyde	4.3 ± 0.5	0.9 ± 0.3	5.2 ± 1.0	1.6 ± 0.8
NADPH/acetaldehyde	5.9 ± 0.6	0.5 ± 0.1	142 ± 21	1.0 ± 0.1
NADPH/acetol	5.1 ± 0.3	0.5 ± 0.2	263 ± 22	1.0 ± 0.1
NADP ⁺ /propan-2-ol	2.2 ± 0.4	1.0 ± 0.2	270 ± 21	0.7 ± 0.1
NAD ⁺ /propan-2-ol	2.6 ± 0.2	65 ± 16	452 ± 21	947 ± 42

Fig. 2. Initial-velocity patterns of the reduction of propan-2-ol by guinea-pig lung carbonyl reductase

(a) Reciprocal plots with NADP⁺ as the varied substrate. Propan-2-ol concentrations were 0.2 mM ($\square$), 0.3 mM ($\bullet$), 0.5 mM ($\triangle$) and 1.0 mM ($\circ$). (b) Reciprocal plots with propan-2-ol as the varied substrate. NADP⁺ concentrations were 2.3 μM ($\square$), 3.5 μM ($\bullet$), 6.6 μM ($\triangle$) and 15 μM ($\circ$). Replots of the slopes ($\circ$) and intercepts ($\bullet$) are shown in the insets. Initial velocity (v) is expressed as units/ml.

reaction, carbonyl reductase oxidized propane-1,2-diol slightly (6% of the acetone reductase activity) but not pyridine-4-methanol, α -methyl-4-nitrobenzyl alcohol or ethanol, suggesting that the equilibrium of these reactions is shifted towards the reductase direction. However, propan-2-ol was oxidized more actively by the enzyme, in contrast with our previous report that no oxidation of this compound was detectable with mouse lung carbonyl reductase [2]. We suspect that a small amount of impurity such as propan-1-ol, which was not oxidized by the lung enzyme, was included in the reagent used previously. Initial-velocity studies carried out in a similar manner with propan-2-ol and NADP⁺ or NAD⁺ (Fig. 2) indicate that a sequential mechanism is also operating in the reverse reaction. The constants for the substrates and cofactors are listed in Table 1.

Product inhibitions

Product-inhibition studies of both the forward and the reverse reactions were carried out to determine the substrate binding order. A summary of the studies of product inhibition in the forward reaction is shown in Table 2, and Table 3 summarizes the results in the reverse reaction. From these data the kinetic mechanism for guinea-pig lung carbonyl reductase appears most likely to be an ordered bi bi mechanism in which NADPH binds first and NADP⁺ leaves last.

This mechanism is similar to those for carbonyl reductases from dog liver [8] and human brain [9], of which the dog liver enzyme has been suggested to undergo cofactor-induced isomerization. The isomerization can be detected with the following relationships between the different constants:

$$\frac{V_f K_i^A}{V_r K_m^A} \geq 1 \quad (2)$$

$$\frac{V_r K_i^Q}{V_f K_m^Q} \geq 1 \quad (3)$$

The failure of either of these equations to hold provides evidence of isomerization of the binary complexes, although validity to the equations does not rule out the isomerization [19,20]. Inserting the values for acetone, propan-2-ol and cofactors from Table 1 into eqns. (2)

Table 2. Product-inhibition patterns and constants in the forward reaction catalysed by guinea-pig lung carbonyl reductase

K_{is} (slope effect) and K_{ii} (intercept effect) values were determined from replots of the slopes and intercepts respectively of double-reciprocal plots with the inhibitors, and are expressed as the means for duplicate analyses.

Product	Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
				K_{is} (μM)	K_{ii} (μM)
NADP ⁺	NADPH	2 mM-Acetone	Competitive	3.6	—
NADP ⁺	Acetone	80 μM -NADPH	Non-competitive	83.6	247
Propan-2-ol	NADPH	2 mM-Acetone	Uncompetitive	—	592
Propan-2-ol	Acetone	80 μM -NADPH	Non-competitive	143	898
NAD ⁺	NADH	20 mM-Acetone	Competitive	970	—
NAD ⁺	Acetone	160 μM -NADH	Non-competitive	2200	2500
Propan-2-ol	NADH	20 mM-Acetone	Uncompetitive	—	1700
Propan-2-ol	Acetone	160 μM -NADH	Non-competitive	4400	1400

Table 3. Product-inhibition patterns and constants in the reverse reaction catalysed by guinea-pig lung carbonyl reductase

The constants were determined and are expressed as described in Table 2.

Product	Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
				K_{is} (μM)	K_{ii} (μM)
NADPH	NADP ⁺	6 mM-Propan-2-ol	Competitive	0.5	—
NADPH	Propan-2-ol	80 μM -NADP ⁺	Non-competitive	20.4	36.9
Acetone	NADP ⁺	6 mM-Propan-2-ol	Uncompetitive	—	226
Acetone	Propan-2-ol	80 μM -NADP ⁺	Non-competitive	246	270
NADH	NAD ⁺	10 mM-Propan-2-ol	Competitive	111	—
NADH	Propan-2-ol	1 mM-NAD ⁺	Non-competitive	349	633
Acetone	NAD ⁺	10 mM-Propan-2-ol	Uncompetitive	—	9800
Acetone	Propan-2-ol	1 mM-NAD ⁺	Non-competitive	9460	12900

and (3), the respective values for that NADH-dependent reaction that hold for the relationships are 110.9 and 2.58, whereas those for NADPH-dependent reaction are 6.95 and 0.23. It should be noted that non-compliance with the relationships has also been observed for a partly random-ordered mechanism of yeast alcohol dehydrogenase [21] or for a simple ordered bi bi mechanism of horse liver alcohol dehydrogenase with commercial NAD⁺ and NADH preparations [22], in which inhibitors are produced during storage [23]. However, the product-inhibition patterns with the present enzyme rule out random association of reactants in both directions, and are consistent with those of ox kidney aldehyde reductase [24] and dog liver carbonyl reductase [8], for which an ordered bi bi mechanism with cofactor-induced isomerization has been suggested. The failure of eqn. (3) to hold was detected with NADP⁺/NADPH as a cofactor, but not with NAD⁺/NADH in spite of the usage of its much higher concentration than that of NADP⁺/NADPH. In a kinetic study of mouse liver 17 β -hydroxysteroid dehydrogenase with the same commercial NADP⁺/NADPH preparations as those used in the present study, the reaction catalysed by the enzyme follows a simple ordered bi bi mechanism [25], and eqns. (2) and (3) hold when the kinetic data for the enzyme are inserted. Therefore the observed inequalities for lung carbonyl reductase may not be due to contamination

of cofactor preparations, and suggest that the reaction catalysed by the enzyme follows a di-iso ordered bi bi mechanism, which is outlined in Scheme 1. This mechanism is in contrast with that of monomeric carbonyl reductase from human brain, for which a simple ordered bi bi mechanism has been proposed [9]. Whether the ordered mechanism with cofactor-induced isomerization is a characteristic solely of oligomeric mammalian carbonyl reductases remains to be determined.

Carbonyl reductase inhibitors

Of the inhibitors for lung carbonyl reductase reported previously [1], pyrazole, benzoic acid and 2-mercaptoethanol inhibited non-competitively with respect to acetone and uncompetitively with respect to NAD(P)H (Table 4), suggesting that these inhibitors bind to the enzyme after cofactor in the reaction mechanism. The inhibition by Cibacron Blue, which is known to bind to many enzymes as a nucleotide analogue [26], was non-competitive with respect to acetone and competitive with respect to NAD(P)H. In addition, benzamide was found to inhibit competitively with respect to acetone and uncompetitively with respect to NAD(P)H. The inhibition patterns by the dead-end inhibitors are consistent with the ordered addition of cofactor followed by carbonyl or alcohol substrate.

I_a is a competitive inhibitor such as Cibacron Blue with respect to A (reduced cofactor) or Q (oxidized cofactor). I_b is a competitive inhibitor such as benzamide with respect to B (carbonyl substrate) or P (alcohol product). I_c is an inhibitor that inhibits uncompetitively with respect to A or Q and non-competitively with respect to B or P.

The inhibition constants were determined as described in Table 2.

Inhibitor	Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
				K_{is} (μM)	K_{ii} (μM)
Pyrazole	NADPH	2 mM-Acetone	Uncompetitive	—	220
	Acetone	80 μM -NADPH	Non-competitive	131	69.8
	NADH	10 mM-Acetone	Uncompetitive	—	228
	Acetone	160 μM -NADH	Non-competitive	—	355
Benzoic acid	NADPH	2 mM-Acetone	Uncompetitive	—	390
	Acetone	80 μM -NADPH	Non-competitive	487	518
2-Mercaptoethanol	NADPH	2 mM-Acetone	Uncompetitive	—	214
	Acetone	80 μM -NADPH	Non-competitive	580	500
Cibacron Blue	NADH	2 mM-Acetone	Competitive	0.2	—
	Acetone	80 μM -NADPH	Non-competitive	16.6	14.6
	NADH	10 mM-Acetone	Competitive	0.1	—
	Acetone	160 μM -NADH	Non-competitive	0.1	0.4
Benzamide	NADPH	2 mM-Acetone	Uncompetitive	—	798
	Acetone	80 μM -NADPH	Competitive	62.0	—
NADPH	NADH	1 mM-4-Nitroacetophenone	Competitive	0.7	—
NADP ⁺	NADH	1 mM-4-Nitroacetophenone	Competitive	0.3	—
NAD ⁺	NADPH	1 mM-4-Nitroacetophenone	Competitive	1930	—

To confirm that the cofactor binds to the free enzyme, the effects of NADPH on the intrinsic fluorescence of the enzyme and that of carbonyl reductase on the fluorescence of NADPH were examined. The fluorescence of the enzyme at 340 nm was quenched by the addition of

The constants of the NADPH- and NADH-dependent reactions were determined to be 16.2 and 11.9

Fig. 3. Fluorescence emission spectra of guinea-pig lung carbonyl reductase and NADPH

(a) Spectra of the enzyme in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.15 M-NaCl at an excitation wavelength of 290 nm. Spectrum A, carbonyl reductase (1.1 μ M); spectrum B, the enzyme plus 50 μ M-acetone; spectrum C, the enzyme plus 2.0 μ M-Cibacron Blue; spectrum D, the enzyme plus 2.0 μ M-NADPH. (b) Spectra of NADPH under the same conditions as (a) with excitation at 340 nm. Spectrum A, the enzyme (1.1 μ M) plus 2.0 μ M-NADPH; spectrum B, NADPH; spectrum C, NADPH plus 0.1 mM-benzamide; spectrum D, the enzyme plus NADPH and benzamide; spectrum E, the enzyme plus benzamide; spectrum F, the enzyme.

respectively. For the ordered mechanism, the Haldane relationship between the different constants is given by:

$$K = \frac{V_f K_i^{\text{NAD(P)}^+} K_m^{\text{propan-2-ol}}}{V_r K_i^{\text{NAD(P)H}} K_m^{\text{acetone}}} \quad (4)$$

Inserting the values in Table 1 into eqn. (4), the K values for NADPH- and NADH-dependent reactions are calculated to be 14.4 and 6.8 respectively. Both values are in acceptable agreement with the values determined experimentally.

REFERENCES

1. Nakayama, T., Hara, A. & Sawada, H. (1982) *Arch. Biochem. Biophys.* **217**, 564–573
2. Nakayama, T., Yashiro, K., Inoue, Y., Matsuura, K., Ichikawa, H., Hara, A. & Sawada, H. (1986) *Biochim. Biophys. Acta* **882**, 220–227
3. Wermuth, B. (1981) *J. Biol. Chem.* **256**, 1206–1213
4. Nakayama, T., Hara, A., Yashiro, K. & Sawada, H. (1985) *Biochem. Pharmacol.* **34**, 107–117
5. Cromlish, J. A. & Flynn, T. G. (1985) *J. Neurochem.* **44**, 1485–1493
6. Molowa, D. T., Wrighton, S. A. & Guzelian, P. S. (1986) *Arch. Biochem. Biophys.* **251**, 487–494
7. Hara, A., Deyashiki, Y., Nakagawa, M., Nakayama, T. & Sawada, H. (1982) *J. Biochem. (Tokyo)* **92**, 1753–1762
8. Hara, A., Nakayama, T., Deyashiki, Y., Kariya, K. & Sawada, H. (1986) *Arch. Biochem. Biophys.* **244**, 238–247
9. Bohren, K. M., von Wartburg, J.-P. & Wermuth, B. (1987) *Biochem. J.* **244**, 165–171
10. Kamitori, Y., Hojo, M., Masuda, R., Inoue, T. & Izumi, T. (1983) *Tetrahedron Lett.* **24**, 2575–2576
11. Beissner, R. S. & Rudolph, F. B. (1978) *Arch. Biochem. Biophys.* **189**, 76–80
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
13. Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
14. Casazza, J. P., Felver, M. E. & Veech, R. L. (1984) *J. Biol. Chem.* **259**, 231–236
15. Argiles, J. M. (1986) *Trends Biochem. Sci.* **11**, 61–63
16. Reichard, G. A., Haff, A. C., Skutches, C. L., Paul, P., Holroyde, C. P. & Owen, O. (1979) *J. Clin. Invest.* **63**, 619–626
17. Owen, O. E., Trapp, V. E., Skutches, C. L., Mozzoli, M. A., Hoeldtke, R. D., Boden, G. & Reichard, G. A. (1982) *Diabetes* **31**, 242–248
18. Johansson, I., Eliasson, E., Norsten, C. & Ingelman-Sundberg, M. (1986) *FEBS Lett.* **196**, 59–64
19. Bloomfield, V., Peller, L. & Albertry, R. A. (1962) *J. Am. Chem. Soc.* **84**, 4375–4381
20. Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, pp. 90–92, Butterworths, London
21. Dickinson, F. M. & Monger, G. P. (1973) *Biochem. J.* **131**, 261–270
22. Dalziel, K. (1963) *J. Biol. Chem.* **238**, 2850–2858
23. Yamauchi, J., Yoshimura, S., Takagahara, I., Fujii, K., Tai, A., Yamashita, J. & Horio, T. (1981) *J. Biochem. (Tokyo)* **90**, 941–955
24. Daly, A. K. & Mantle, T. J. (1982) *Biochem. J.* **205**, 381–388
25. Hara, A., Nakayama, T., Nakagawa, M., Inoue, Y., Tanabe, H. & Sawada, H. (1987) *J. Biochem. (Tokyo)* **102**, 1584–1592
26. Thompson, S. T., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 669–672

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