Biochimica et Biophysica Acta, 567 (1979) 66—74
© Elsevier/North-Holland Biomedical Press

BBA 68688

MECHANISM OF INHIBITION OF MALATE DEHYDROGENASE BY THYROXINE DERIVATIVES AND REACTIVATION BY ANTIBODIES

HOMOGENEOUS ENZYME IMMUNOASSAY FOR THRYROXINE *

EDWIN F. ULLMAN, ROBERT A. YOSHIDA, JUDITH I. BLAKEMORE, EDWARD MAGGIO and RICHARD LEUTE

Syva Research Institute, 3221 Porter Drive, Palo Alto, CA 94304 (U.S.A.)

(Received August 4th, 1978)

Key words: Malate dehydrogenase; Inhibition mechanisms; Thyroxine derivative; Antibody reactivation; Immunoassay

Summary

Pig heart mitochondrial malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37) is about 90% inhibited upon labelling an average of two amino groups per subunit with an active ester of thyroxine. Inhibition is probably associated primarily with thyroxine binding to one specific group which is normally unreactive but becomes activated upon noncovalent binding of thyroxine derivatives to the enzyme. Enzyme inhibition is due to a decrease in the rate of association of NAD. Antibodies to thyroxine induce a slow conformational change with partial reversal of inhibition of more heavily labelled conjugates. The antibody-induced activation is not cooperative and does not require bivalent association of the antibody. Activation can be blocked by the presence of free thyroxine and is the basis for a clinically useful assay for serum thyroxine.

Introduction

Certain enzymes, when covalently conjugated with low molecular weight haptens, can be inhibited by antihapten antibodies. Among others, lysozyme [1,2], malate dehydrogenase (L-malate-NAD$^+$ oxidoreductase, EC 1.1.1.37) [3,4], and glucose-6-phosphate dehydrogenase [5] show this behavior. Inhibition of enzyme activity by anti-hapten antibodies can be blocked by prior

* Contribution No. 67 from Syva Research Institute.

incubation of the antibodies with the free hapten. This provides the basis for homogeneous enzyme immunoassays which enjoy wide clinical use because of the speed and ease of automation afforded by the absence of any separation steps [6,7].

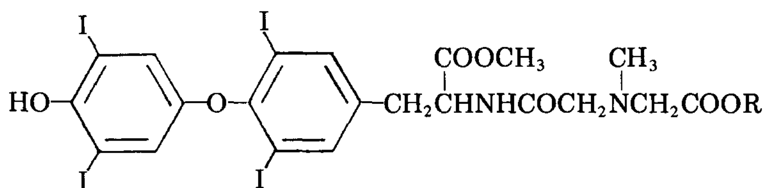
During the development of such an assay for thyroxine it was noted that conjugation of thyroxine derivatives to malate dehydrogenase led to extensive loss of enzyme activity and that, unexpectedly, anti-thyroxine antibodies partially reversed the inhibition. We describe here observations which provide insight into the mechanism of inhibition and antibody-induced activation, and which permit the construction of a clinically useful assay for serum thyroxine [8,9].

Experiment procedures

Materials

Pig heart mitochondrial malate dehydrogenase from Miles (Elkhart, ID), after purification by the procedure of Glatthaar et al. [10], had a specific activity of 355 IU/mg and $E^{1\%}_{278nm}$ 2.43 ± 0.06. Thyroxine was from Sigma (St. Louis, MO), [2-^{14}C]thyroxine from New England Nuclear (Worcester, MA), *N*-succinimidyl 3-(4-hydroxyphenyl) propionate from Pierce (Rockford, IL), and *N*-methyliminodiacetic acid, dicyclohexylcarbodiimide and *N*-hydroxysuccinimide from Aldrich (Milwaukee, WI).

Methyl thyroxinate hydrochloride was prepared by allowing thyroxine in dry methanol saturated with HCl gas to stand overnight at room temperature. The nearly pure ester precipitated in 91—96% yield.



Compound 1 R=H

Compound 2 R=succinimid-1-yl

Compound 1 was prepared from 320 mg *N*-methyliminodiacetic acid anhydride and 150 mg methyl thyroxinate hydrochloride in 4.5 ml dry tetrahydrofuran/dimethylformamide (Me_2NCHO) (1 : 1, v/v) containing 0.30 ml triethylamine. After 30 min at 25°C, H_2O was added and the white precipitate recrystallized from hexane/ethyl acetate (45% yield). Additional purification was by thin-layer chromatography on silica gel (developed in triethylamine/ CH_3OH/CH_2Cl_2, 0.2 : 1 : 10, v/v).

Compound 2 was prepared from 44 mg dicyclohexylcarbodiimide, 180 mg compound 1 and 26 mg *N*-hydroxysuccinimide in 5 ml dry tetrahydrofuran at 2—4°C. After stirring overnight at 2—4°C, the product was isolated in 81—91%

yield by chromatography with *n*-hexane/CH_2Cl_2 (1 : 5, v/v) on cellulose and recrystallization from CH_2Cl_2/petroleum ether.

Enzyme conjugates were prepared by stepwise addition of 1—5 μl aliquots of 24 mM compound 2 in Me_2NCHO to a stirred solution of 8 mg enzyme in about 1 ml 50 mM $NaHCO_3/Na_2CO_3$ (pH 9.1) buffer containing 25% Carbitol® at 2—4°C. The reaction was not instantaneous, and sequential additions were made in 15 min intervals to ensure complete reaction. The enzyme activity was monitored by dilution of aliquots of the reaction mixture into an assay solution. The conjugates were purified by dialysis against 1 M K_2HPO_4, 1 mM NaN_3 and 1 mM EDTA, at 2—4°C followed by gel filtration with Sephadex® G-50M, using the same buffer. Yields of labelling were near quantitative. The number of groups bound to the enzyme were determined by radioactivity and Lowry protein determination [11]. For studies of the effect of the concentration of compound 2 the procedure was unchanged except the volumes of the enzyme solutions were increased by diluting up to 100-fold.

Prelabelling of enzyme with *N*-succinimidyl 3-(4-hydroxyphenyl)-propionate was carried out in a similar fashion with 1.4 equivalents of the ester per subunit. Immediately after the final addition and incubation, aliquots of compound 1 in Me_2NCHO were added as in the preceding procedure.

Hydroxylamine treatment of enzyme conjugates was carried out by incubation with 1 M hydroxylamine at pH 9.2 for 40 min at 0—2°C followed by dialysis and gel filtration, as described above.

Enzyme kinetics were determined at 30.0 ± 0.2°C by measuring the steady-state rate of appearance of NADH at 340 nm with a Bausch and Lomb Spectronic 100 spectrophotometer. The steady-state rates of 0.01—0.1 *A*/min remained constant during a 3—8 min period after mixing. The reactions proceeded to less than 0.3% of completion and were initiated by combining 1—10 μl enzyme; 1.9 ml 94.7 mM glycine/NaOH (pH 9.5), 0.095% rabbit albumin, 0.0095% EDTA, 5.26 mM NAD; 0.1 ml 2 M L-malic acid (pH 9.5); and 25 μl antibody solution or antibody buffer. Measurements in the presence or absence of excess antibody gave identical rates whether enzyme or malate was added last to a preincubated mixture (15 min) of the other components. The latter order of addition was employed for the determination of kinetic parameters; the kinetics of enzyme activity were followed for all possible orders of additions using a Cary 15 spectrophotometer; and the enzyme was added last for all other studies.

Thyroxine antibodies were produced using a conjugate of bovine albumin prepared similarly to the enzyme conjugates. Sheep were immunized by bimonthly subcutaneous and intramuscular injections with an emulsion of the conjugate, 0.9% saline, and complete Freund's adjuvant. The IgG fraction of the antiserum obtained by precipitation with 50% saturated ammonium sulfate and DEAE-cellulose chromatography, was stored in 10 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 1% KCl, 1 mM NaN_3, 1 mM EDTA.

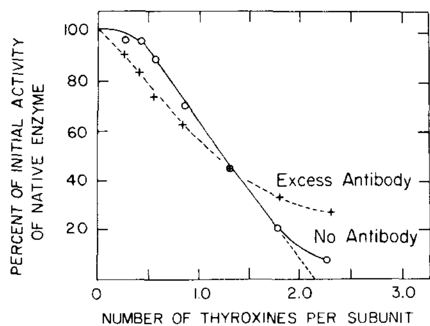
Fab fragments of anti-thyroxine IgG were prepared by a modification of the procedure of Porter [12].

Results

Reaction of malate dehydrogenase with compound 2 caused strong inhibition of enzyme activity. Binding of about two thyroxines per subunit produced over 90% inhibition (Fig. 1). Addition of excess anti-thyroxine antibody to the conjugates led to either slightly increased inhibition or partial reversal of the inhibition depending on the extent of conjugation (Fig. 1). Only those conjugates with greater than 1.2—1.5 thyroxines per enzyme subunit (35 000 daltons [13,14]) were activatable by excess antibody. Treatment of the conjugates with 1 M hydroxylamine to remove any tyrosine-bound thyroxines [15] led to no loss of thyroxine or change in enzyme activity.

These results suggested that labelling occurs moderately selectively at two amino groups per subunit, and that a preferentially labelled group might react to yield antibody-inhibitable conjugate while a second group might give antibody-activatable conjugate on labelling. This was tested by prelabelling the enzyme prior to thyroxine labelling with 1.4 equivalents of a compound structurally related to thyroxine, 3-(4-hydroxyphenyl)-propionate. The prelabelled enzyme, which was 23% more active than the native enzyme, was initially more inhibited per equivalent of bound thyroxine, and about 90% inhibition was again observed with two bound thyroxines (Fig. 2). Moreover only 0.7 thyroxines per subunit was now sufficient to cause antibody induced activability.

The possibility that the high efficiency of inhibition by compound 2 was due to affinity labelling was examined by studying the activity of conjugates prepared with different concentrations of compound 2. With increasing concentrations, saturation of affinity labelling sites should occur and thus less selective labelling should take place. However, on labelling with 1, 10 and 100 μM compound 2 and stopping each reaction when the enzyme activity had decreased by 87%, the resulting conjugates had identical thyroxine to enzyme



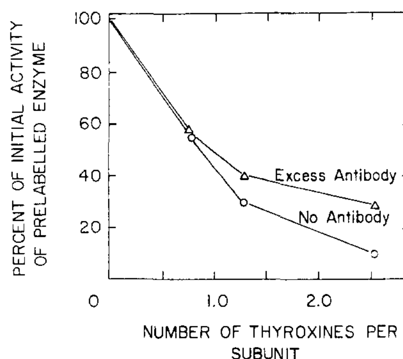


Fig. 1. Activity of malate dehydrogenase on titration with compound 2. Rate measurements were made prior to Sephadex separation of reactants using either non-specific sheep IgG (○) or sheep anti-thyroxine antibodies (+) in the antibody buffer (see Enzyme Kinetics).

Fig. 2. The activity of malate dehydrogenase that had been prelabelled with about 1.4 equivalents 3-(4-hydroxyphenyl) propionyl groups per subunit was followed during titrations with compound 2. Rate measurements were made prior to Sephadex separations of reactants using either non-specific sheep IgG (○) or sheep anti-thyroxine antibodies (△) in the antibody buffer (see Enzyme Kinetics).

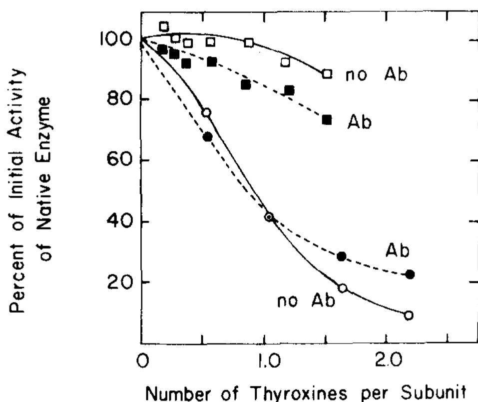


Fig. 3. Activity of malate dehydrogenase during titration with compound 2 in the presence of 1.3 mM compound 1 (○, ●) or 2.4 mM NAD and 9.7 mM oxaloacetate (□, ■). Rate measurements were made prior to Sephadex separation of reactants using either non-specific sheep IgG ——— or sheep anti-thyroxine antibodies - - - - - - in the antibody buffer (see Enzyme Kinetics).

subunit ratios (1.6) and were identically activatable by antibody (180 ± 12%).

The use of substrates to protect the labelling sites was examined by reaction of the enzyme with compound 2 in the presence of NAD and oxaloacetate. Under these conditions there was a small but reproducible increase in enzyme activity (2.3%) which then gradually dropped off with increasing labelling, and the effect of antibody on all the conjugates was now weakly inhibitory (Fig. 3). The presence during the conjugations of the competitive inhibitor, compound 1 [16], failed to provide similar protection against inhibition by compound 2 (Fig. 3).

Titration of enzyme conjugates with anti-thyroxine antibody produced a linear increase in activity over the first 40% of the activation range, and linear Scatchard plots were obtained. Fab fragments behaved similarly. The antibody-

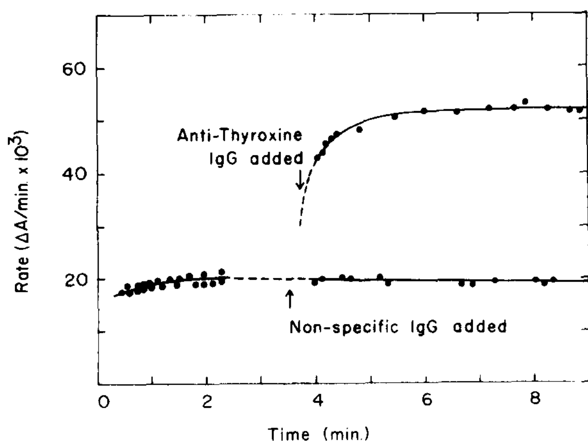


Fig. 4. Increase in catalyzed rate upon diluting 2 μl 1.0 M K_2HPO_4, $3.3 \cdot 10^{-6}$ M enzyme conjugate with 1.9 thyroxines per subunit into 1.0 ml, 90 mM glycine (pH 9.5), 5.5 mM NAD, 100 mM malate. Excess (2 μl) anti-thyroxine sheep antibody or non-specific sheep IgG was added as shown. The upper curve has been extrapolated using the first order rate constant to the time of addition of anti-thyroxine.

TABLE I

KINETIC CONSTANTS AT 30°C FOR ENZYME LABELLED WITH 2.4 MOLECULES OF THYROXINE DERIVATIVE 1 PER SUBUNIT

Double-reciprocal plots of velocity versus one substrate concentration were constructed for various concentrations of the second substrate. Secondary plots of the slopes and of the $1/v$ intercepts against the reciprocals of the second substrate concentrations were linear. K_m, V, and K_{NAD} (the dissociation constant for NAD) were derived from the latter plots from the equations by Segel [23] for compulsory ordered mechanisms. The bimolecular rate constant for binding NAD, k_1, was taken as $V/K_{m(NAD)}$ [23].

	$K_{m(mal)}$ (mM)	$K_{m(NAD)}$ (mM)	K_{NAD} (mM)	V relative	k_1 relative
Native enzyme	0.30	0.13	0.69	25	224
Conjugate	21.	1.17	1.0	1	1
Conjugate + Ab	1.16	0.06	0.31	6.2	120

induced activation was reversed by an amount directly related to the concentration of added free thyroxine. This permitted an assay to be set up that was sufficiently sensitive to permit the determination of thyroxine in serum (30—300 nM).

The kinetic constants for native and conjugated enzyme in the presence and absence of antibody are compared in Table I. Conjugation led to increases in K_m for both substrates, a decrease in the rate of association of NAD (k_1), but little change in the NAD dissociation constant (K_{NAD}) relative to native enzyme. Antibody largely reversed these changes.

The kinetic measurements were complicated by a gradual rise in activity of the conjugates after initiation of the reaction (Fig. 4). These rate changes were associated with dilution of stock solutions of the conjugates (pH 9.2, 1.0 M phosphate) into the assay medium (pH 9.5, 0.1 M glycine plus substrates). They did not occur with native enzyme nor when the conjugates were stored in pH 9.5, 0.1 M glycine. However, with antibody present, the slow activation of the conjugates occurred with no change in buffer and was observed no matter whether enzyme conjugates, substrates, or antibody were added last to preincubated mixtures of the other components. The kinetics of activation were typical of a reversible unimolecular conversion from a species of low catalytic activity to a more active species. The log of the difference between the measured rate and the final steady-state rate fell linearly with time, and the first order rate constant of 1.2 ± 0.5 min^{-1} was independent of the antibody or enzyme concentrations or the order of addition of the reactants.

Discussion

Pig heart mitochondrial malate dehydrogenase has two identical subunits of about M_r = 35 000 [13,14]. It is reported to bind two molecules of NADH [17] and has been suggested to function by a compulsory ordered mechanism [18, 19]. A variety of electronegatively substituted phenols including thyroxine act as reversible inhibitors [20]. Thyroxine has been shown to be a competitive inhibitor versus DNA (K_i; 8.5 μM) and derivatization of the carboxyl or amino groups of thyroxine has little effect on its inhibitory properties [16,21].

Location of inhibitory sites. Labelling of the enzyme with an active ester at pH 9.1 should lead only to modification of amino and tyrosine hydroxyl groups. The stability of the conjugates towards hydroxylamine suggests that labelling occurs only on amino groups [15]. The strong inhibition produced by labelling two amino groups per subunit with thyroxine stands in contrast to only 50% inhibition on labelling eight amino groups with morphine [3]. Thus compound 2 reacts with exceptional selectively. Moreover, inhibition of greater than 50% on labelling the prelabelled enzyme with one thyroxine per subunit suggests that labelling of a single specific group may be sufficient to cause complete inhibition. Since the prelabelled enzyme retained its full capacity for inhibition (>90%) by compound 2 (Fig. 2), blocking of this inhibitory group during prelabelling does not occur. Nevertheless, based on the slopes of the inhibition curves (Figs. 1 and 2), there must be competitive labelling at groups that are non-inhibitory, one of which is probably more reactive than the inhibitory amino group. These groups are partially blocked during prelabelling.

The exceptional selectivity of compound 2 must be caused either by affinity labelling or else by reaction with an amino group that becomes hyper-reactive due to a reagent-induced conformational change. The observed protection from inhibition by NAD and oxaloacetate, which form a ternary complex with the enzyme [22], could be explained by either mechanism. On the other hand, thyroxine and compound 1 are both competitive inhibitors of the native enzyme with like inhibition constants [16], and it is probable that compound 2 binds similarly. Thus, if compound 1 competes with compound 2 for binding sites it would prevent affinity labelling but would not interfere with a thyroxine-induced conformational change. The slight effect of compound 1 on inhibition by compound 2 (Fig. 3) therefore excludes affinity labelling. Conjugations that produced unchanged labelling specificity with varying concentrations of compound 2 likewise are inconsistent with this labelling mechanism.

Activation of enzyme conjugates by antibody. The strong inhibition of activity and increase in K_m produced by compound 2 may arise from interaction of the bound thyroxine with an inhibitory effector site. The antibody-induced reversal of inhibition and decrease in K_m may then be explained by disruption of this interaction. However, the observed strict linear increase in activity of the conjugates with added antibody requires that any such interaction must be very weak. Strong non-covalent association of the inhibitory thyroxines would reduce antibody affinity selectively towards these groups and would therefore produce a sinusoidal rather than linear response.

The linearity of the Scatchard and direct plots obtained by titrating the conjugates with antibody reflects the homogeneity of the conjugates rather than the antibody since the conditions were limited to hapten excess. These data not only require that each conjugated thyroxine has a similar binding affinity toward antibody but that enzyme activation is not dependent on simultaneous binding by several antibodies. Similar behavior by Fab fragments eliminates the possibility that bivalent attachment by antibody is necessary to effect activation.

The non-instantaneous increase in the enzyme rate with added antibody or on changing buffer (Fig. 4) suggests that the conjugates undergo a slow conformational change between species having different catalytic activity. Alterna-

tive interpretations such as slow bimolecular reaction of enzyme with antibody can be ruled out by the occurrence of slow activation when substrates are added to pre-incubated mixtures of enzyme and antibody. Furthermore, neither dimerization nor dissociation of enzyme aggregates into smaller units is consistent with the independence of the first order rate constant for activation with changes in enzyme and antibody concentrations.

Mechanism of inhibition. The simplest model for inhibition of malate dehydrogenase by compound 2 requires that one specific inhibitory amino group per subunit becomes activated upon noncovalent binding of a thyroxine. Thyroxine bound to this group does not significantly change the enzyme affinity for NAD but does reduce the rate of binding (k_1) of this substrate. Thus conformational rather than steric or competitive binding effects play a predominant role in enzyme inhibition. Moreover, the kinetics of activation of the conjugates require that there is a slow conformational equilibrium which can by shifted upon binding by antibody. This phenomenon may be caused by a very weak noncovalent interaction of bound thyroxine with the enzyme which is disrupted by antibody binding, although alternative mechanisms for conformational control cannot be eliminated.

Enzyme immunoassay for thyroxine. The inhibition of antibody-induced activation of enzyme conjugates by free thyroxine demonstrates the specificity of activation by antibody. Inhibition by thyroxine cannot be explained by direct thyroxine inhibition of the enzyme since the thyroxine concentrations employed were much below K_i. The effect of thyroxine on the enzyme activity affords the opportunity to construct a simple assay for thyroxine which requires only the sequential mixing of the thyroxine sample, antibody and substrate solution, and the enzyme conjugate. Satisfactory clinical data have been obtained when using a modification of this procedure in which serum samples are pretreated with sodium hydroxide to destroy endogenous enzyme activity and release thyroxine from thyroxine binding globulin [8,9].

Acknowledgement

The authors gratefully acknowledge the help of Wendy Eimstad, Terry Ann Houk and Roberta Ernst for evaluation of antibodies and technical assistance, and the help of Dan Leung, Robert Ronald, Bernard Sheldon and Mae Hu in the synthesis of thyroxine derivatives.

References

1 Rubenstein, K.E., Schneider, R.S. and Ullman, E.F. (1972) Biochem. Biophys. Res. Commun. 47, 846—851
2 Schneider, R.S., Lindquist, P., Wong, E.T., Rubenstein, K.E. and Ullman, E.F. (1973) Clin. Chem., 19, 821—825
3 Rowley, G.L., Rubenstein, K.E., Huisjen, J. and Ullman, E.F., (1974) J. Biol. Chem. 250, 3759—3766
4 Rowley, G.L., Armstrong, T.A., Crowl, C.P., Eimstad, W.M., Hu, M.W., Kam, J.K., Rodgers, R., Ronald, R.C., Rubenstein, K.E., Sheldon, B.G. and Ullman, E.F. (1976) in Cannabinoid Assays in Humans (Willette, R.E., ed.), pp. 28—32, NIDA Research Monograph 7, HEW, Rockville, MD
5 Rowley, G.L. Rubenstein, K.E., Weber, S.P. and Ullman, E.F., (1976) Abstracts 172nd Amer. Chem. Soc. Meeting BIOL 151, Port City Press, Baltimore, MD
6 Scharpe, S.L., Cooreman, W.M., Bloome, W.J. and Lackeman, G.M., (1976) Clin. Chem. 22, 733—738
7 Wisdom, G.B. (1976) Clin. Chem. 22, 1243—1255

8 Ullman, E.F., Blakemore, J., Leute, R.K., Eimstad, W. and Jaklitsch, A., (1975) Clin. Chem. 21, 1011
9 Galen, R.S. and Forman, D. (1977) Clin. Chem. 23, 119—121
10 Glatthaar, B.E., Barbarash, G.R., Noyes, B.E., Banaszak, L.J. and Bradshaw, R.A. (1974) Anal. Biochem. 57, 432—451
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Porter, R.R. (1959) Biochem. J. 73, 119—126
13 Noyes, B.E., Glatthaar, B.E., Garavelli, J.S. and Bradshaw, R.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1334—1338
14 Thorne, C.J.R. and Kaplan, N.O. (1963) J. Biol. Chem. 238, 1861—1868
15 Riordan, J.F., Vallee, B.L. and Saunders, D.M. (1963) Biochemistry 2, 1460—1467
16 Maggio, E. and Ullman, E.F. (1978) Biochim. Biophys. Acta 522, 284—290
17 Holbrook, J.J. and Wolfe, R.G. (1972) Biochemistry 11, 2499—2502
18 Harada, K. and Wolfe, R.G. (1968) J. Biol. Chem. 243, 4131—4137
19 Silverstein, E. and Sulebele, G. (1969) Biochemistry 8, 2543—2550
20 Wedding, R.T., Hausch, C. and Fukuto, T.R. (1967) Arch. Biochem. Biophys. 121, 9—21
21 Wolff, J. and Wolff, E.C. (1957) Biochim. Biophys. Acta 26, 387—396
22 Raval, D.N. and Wolfe, R.G. (1963) Biochemistry 2, 220—224
23 Segel, I.H. (1975) Enzyme Kinetics, pp. 560—593, John Wiley and Sons, New York, NY