Cytochrome b_5 Activation of CYP3A4 Activity is Buffer and Reductase Dependent.

Gillian M. Macintyre & Michael W. Voice

Cypex Ltd., 6 Tom McDonald Avenue, Dundee, DD2 1NH, Scotland

Abstract

The ability of cytochrome b_5 to increase the activity of CYP3A4 when included in reconstitution experiments with purified NADPH P450 reductase (reductase) has been well documented. There is little data, however, on the ability of cytochrome b_5 to affect the activity of CYP3A4 when CYP3A4 is co-expressed with NADPH P450 reductase in membrane preparations from E. coli. We have investigated the effect of adding recombinant human cytochrome b_5 to preparations of E. coli membranes containing co-expressed human CYP3A4 and human NADPH P450 reductase (Bactosomes) in different assay buffers.

We have demonstrated that the effect of cytochrome b_5 is buffer dependent with the most activation of CYP3A4 being seen when 50 mM HEPES is used as the assay buffer compared to 100 mM phosphate. We are able to alter the reductase : CYP3A4 ratio by using different co-expression systems. By adding cytochrome b_5 to membrane preparations with differing levels of reductase relative to CYP3A4 we have shown that this can affect the action of cytochrome b_5. We found that, where there is a high reductase : P450 ratio, the activity of the CYP3A4 is not significantly increased in 100 mM phosphate buffer and is only slightly (30%) increased in 50 mM HEPES buffer. When the ratio of reductase : CYP3A4 is reduced then the addition of cytochrome b_5 results in a much bigger increase in testosterone turnover by CYP3A4 in 50 mM HEPES buffer (approx 100%) and a small but significant increase in 100 mM phosphate buffer.

Introduction

It is well documented that the inclusion of cytochrome b_5 in reconstitution mixes with CYP3A4 can significantly increase the turnover of some substrates (eg. Yamazaki, H. *et al* (1995)). These studies used systems where purified proteins (CYP3A4, NADPH P450 reductase and cytochrome b_5 have been reconstituted in an artificial phospholipid mix. In general, the activity of CYP3A4 is much lower in systems where purified CYP3A4 and NADPH P450 reductase are reconstituted compared to systems where the two proteins are co-expressed and allowed to associate in the membrane *in vivo*. Although, in one study, cytochrome b_5 has been shown to increase testosterone turnover by CYP3A4 when co-expressed in *E. coli* together with NADPH P450 reductase (Voice, M. *et al* (1999) there is little additional information available regarding the effects exerted by cytochrome b_5 when the CYP3A4 and reductase have been produced in this way. Here we have further investigated the effects of cytochrome b_5 on CYP3A4 activity in bacterial membranes containing co-expressed CYP3A4 and human NADPH P450 reductase.

Fig 1. Effect of the addition of cytochrome b_5 on the Vmax for testosterone turnover by CYP3A4 Bactosomes.

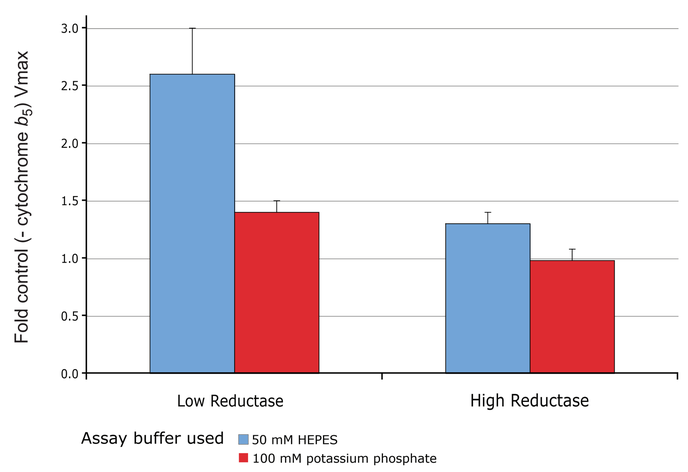


Fig 2. Effect of the addition of cytochrome b_5 on the Km for testosterone of CYP3A4 Bactosomes.

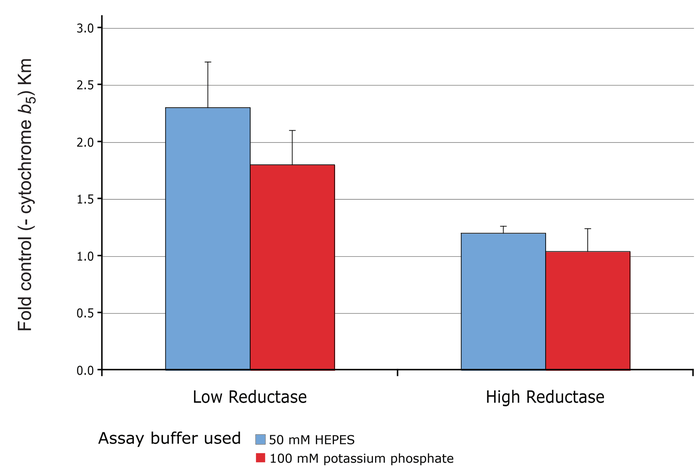


Fig 3. Effect of the addition of cytochrome b_5 on nifedipine turnover by CYP3A4 Bactosomes.

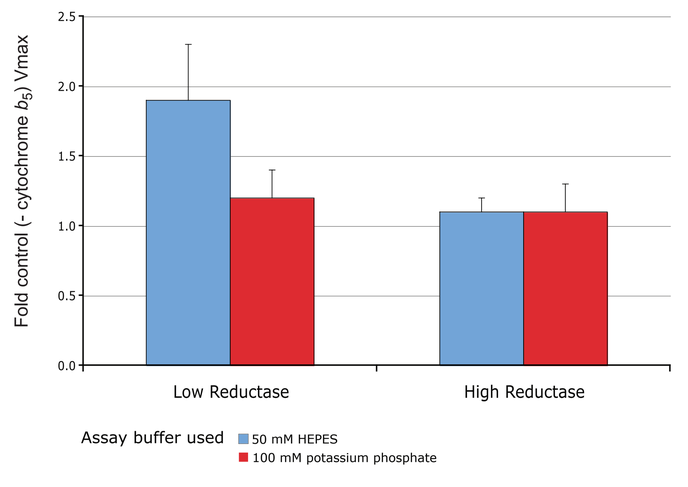
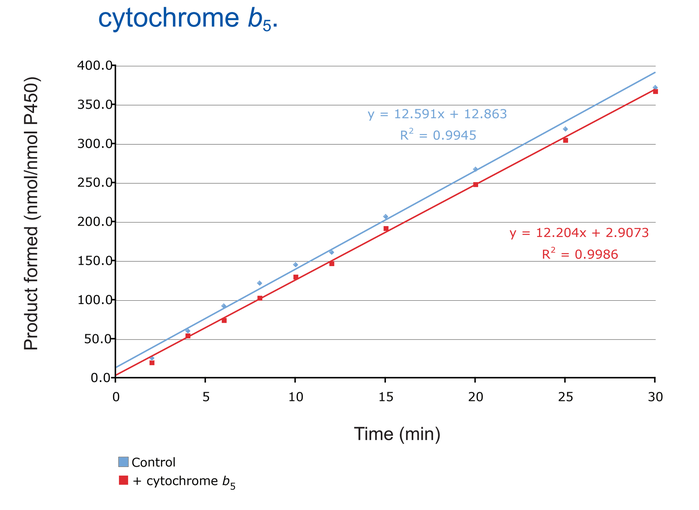


Fig 4. Time Linearity of testosterone turnover by CYP3A4LR Bactosomes with and without cytochrome b_5.



Materials & Methods

All chemicals were obtained from Sigma-Aldrich or VWR International unless otherwise indicated. Recombinant human CYP3A4 co-expressed in *E. coli* with human NADPH P450 reductase (Bactosomes) 7-benzyloxyquinoline (7-BQ) and 7-hydroxyquinoline were supplied by Cypex Ltd.

Cytochrome b_5 was expressed in *E. coli* as previously described (Voice, M. *et al* (1999) and purified using a nickel agarose column obtained from GE Healthcare Ltd.

CYP3A4 activity was determined using testosterone, nifedipine or 7-benzyloxyquinoline (7-BQ) as substrate at 37°C for 5 minutes in the buffer specified (total assay volume 1.0 ml (0.2 ml 7-BQ)). The buffers used were either 100 mM potassium phosphate pH 7.4 or 50 mM potassium HEPES pH 7.4. Both buffers contained 5 mM $MgCl_2$. The substrate concentration ranges used were 40 - 200 µM (testosterone), 70 µM (7-BQ, high reductase 3A4), 200 µM (7-BQ, low reductase 3A4) and 200 µM (nifedipine). For testosterone and nifedipine, the reactions were started by the addition of 200 µl 5x NADPH generating system (5 mM NADPH, 25 mM glucose-6-phosphate, 5 U/ml glucose-6-phosphate dehydrogenase). Reactions were stopped by the addition of 125 µl 1 M HCl . Samples were centrifuged at 13,000 rpm in a microfuge and the supernatants analysed by reverse phase HPLC with UV detection. 7-BQ turnover was initiated by the addition of 40 µl 5 x NADPH generating system and generation of metabolite (7-hydroxyquinoline) was measured by continuous monitoring of fluorescence (λ_{ex} 430 nm, λ_{em} 530 nm). All assays were performed on at least three different preparations of *E. coli* membranes.

Results

Purified cytochrome b_5 was added to *E. coli* membranes containing co-expressed CYP3A4 and NADPH P450 reductase in a 5 fold excess over the CYP3A4. This resulted in an increase in testosterone turnover by CYP3A4 (Fig. 1) when HEPES was used as the assay buffer. This increase in activity was most marked when the assay was carried out using membranes with a reductase : P450 ratio of 0.3 (Low reductase Bactosomes) where the Vmax for testosterone turnover increased from 1.2 nmol/min/nmol P450 to 2.6 nmol/min/nmol P450. When the reductase : P450 ratio was increased to 1.5 (High reductase Bactosomes), the effect of adding cytochrome b_5 diminished. When 100 mM potassium phosphate was used as the assay buffer, the effect of adding cytochrome b_5 to CYP3A4 was reduced and, when High reductase Bactosomes were used, the addition of cytochrome b_5 had no effect on testosterone turnover.

In Low reductase CYP3A4 Bactosomes, the Km for testosterone increased with the addition of cytochrome b_5 when either 50 mM HEPES or 100 mM potassium phosphate was used as the assay buffer (Fig.2). In High reductase Bactosomes, there was no change in the Km for testosterone with the addition of cytochrome b_5 regardless of the assay buffer used.

The effect of adding cytochrome b_5 on nifedipine oxidation by CYP3A4 was determined. With Low reductase CYP3A4, the addition of cytochrome b_5 led to an increase in nifedipine oxidation in both 50 mM HEPES and 100 mM phosphate buffer with the effect being more marked in 50 mM HEPES (1.8 fold control compared to 1.2 fold in 100 mM phosphate). The inclusion of cytochrome b_5 had no effect on the rate of nifedipine oxidation by High reductase CYP3A4. Overall, the effects of adding cytochrome b_5 were less marked when nifedipine was used as substrate compared to the effects seen with testosterone.

The effects of adding cytochrome b_5 to CYP3A4 containing membranes were also determined using a third substrate, 7-benzyloxyquinoline. With this substrate, the activity of CYP3A4 was unaffected by the addition of cytochrome b_5 in both High and Low Reductase preparations (data not shown).

The time for which testosterone turnover was linear was determined (Fig. 4). We have shown (see poster 142) that, as the activity of CYP3A4 is increased, either by increasing the amount of NADPH P450 reductase, or by changing the assay buffer used, the time for which the reaction is linear decreases. In contrast, with the addition of cytochrome b_5 to Low reductase CYP3A4, although there was a significant increase in activity, there was no reduction in the time for which testosterone turnover was linear.

Conclusions

- The effect of cytochrome b_5 on CYP3A4 activity is buffer dependent, CYP3A4 activity is increased more in 50 mM HEPES buffer than in 100 mM phosphate buffer
- The effect of cytochrome b_5 on CYP3A4 activity is dependent on the relative amount of NADPH P450 reductase; cytochrome b_5 has a much smaller effect on CYP3A4 activity in bacterial membranes with a higher level of NADPH P450 reductase.
- Activation of CYP3A4 by the addition of cytochrome b_5 does not result in a reduction of the length of time for which testosterone turnover is linear.

References

Yamazaki, H., Ueng, Y-F., Shimada, T. & Guengerich, F.P. (1995) Biochemistry **34** pp 8380 - 8389
Roles of divalent metal ions in oxidations catalysed by recombinant cytochrome P450 3A4 and replacement of NADPH-Cytochrome P450 reductase with other flavoproteins, ferredoxin, and oxygen surrogates.

Voice, M., Zhang, Y., Wolf, C.R., Burchell, B. & Friedberg, T. (1999) Arch. Biochim. Biophys. **366** (1) pp 116-124
Effects of human cytochrome b_5 on CYP3A4 activity and stability *in vivo*.