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A simple method for purification of rat hepatic microsomal cytochrome b_5

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Rat hepatic microsomal cytochrome b_5 was purified to homogeneity by solubilization with the detergent Lubrol 12-A9 and chromatography on Fractogel TSK DEAE-650(S). The protein was obtained in high yield (52–87%) in 8h, and only one polypeptide band, of M_r 16600, was visible after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Hepatic microsomal cytochrome b_5 is a membrane-bound protein, and its role *in vivo* is to provide electrons for a host of important biosynthetic reactions (Oshino *et al.*, 1971; Paltauf *et al.*, 1974; Reddy *et al.*, 1977; Keyes *et al.*, 1979; Von Jagow & Sebald, 1980; Grinstead & Gaylor, 1982; Strittmatter *et al.*, 1982). Cytochrome b_5 also plays an important role in the biotransformation of several drugs (Imai & Sato, 1977; Sugiyama *et al.*, 1979; Ingelman-Sundberg & Johansson, 1980; Canova-Davis & Waskell, 1984).

Pure cytochrome b_5 has been obtained from pig, horse, ox, rabbit and rat liver (for a review, see Von Jagow & Sebald, 1980). All the widely used purification procedures for cytochrome b_5 are technically difficult and involve complex solubilization procedures followed by several chromatography steps over a period of days (see Spatz & Strittmatter, 1971; Ozols, 1974; Chiang, 1981; Kamataki *et al.*, 1981).

In this paper I report an extremely simple method for the purification of cytochrome b_5 in high yield within 8h.

Materials and methods

Fractogel TSK DEAE-650(S) 'Merck' was purchased from BDH Chemicals, Poole, Dorset, U.K. Lubrol 12A-9 (a condensate of dodecyl alcohol with approx. 9.5mol of ethylene oxide/mol) was from ICI Organics Division, Manchester, U.K. Protein standards used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were purchased from Boehringer, Mannheim, West Germany, and Sigma (London) Chemical Co., Poole, Dorset, U.K. All other chemicals were analytical-reagent grade.

Preparation of the liver microsomal fraction

A microsomal fraction was prepared from 10g of liver from starved male Wistar rats as previously described (Arion *et al.*, 1984).

Gel electrophoresis

Slab gradient (7–16%) and non-gradient (11%) polyacrylamide-gel electrophoresis was performed at 20°C in the presence of 0.1% sodium dodecyl sulphate as described by Laemmli (1970). The M_r of the purified cytochrome b_5 was determined on an 11%-acrylamide gel by comparison with the mobility of bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soya-bean trypsin inhibitor (20100), lactalbumin (14200) and cytochrome *c* (12500).

Measurement of cytochrome b_5

The concentration of purified cytochrome b_5 was ascertained from the absolute spectrum of the ferric protein by using an absorption coefficient of $117\,mM^{-1}\cdot cm^{-1}$ at 413nm (Strittmatter & Velick, 1956). In crude preparations the amount of cytochrome b_5 was estimated from the reduced-minus-oxidized spectra (Estabrook & Werringloer, 1978; Ozols, 1974). Protein concentrations were determined by the method of Bradford (1976).

Results

Purification of rat hepatic microsomal cytochrome b_5

Liver microsomal pellets were resuspended at a protein concentration of 5mg/ml in 1% Lubrol/5mM-Hepes/20mM-NaF/62.5mM-sucrose, pH7.4, by gentle homogenization at 4°C. This

preparation was centrifuged at 105000*g* for 60min. The Lubrol supernatant was applied to a Fractogel TSK DEAE-650(S) column (45cm × 2cm) previously equilibrated in 5mM-Hepes/20mM-NaF/20% glycerol/0.05% Lubrol, pH7.4. After elution with this solution, until protein could not be detected spectrophotometrically at 280nm, the column was washed with 5mM-Hepes/20mM-NaF/20% glycerol/70mM-$(NH_4)_2SO_4$, pH7.4. Two protein peaks were then eluted with 5mM-Hepes/20mM-NaF/20% glycerol/70mM-$(NH_4)_2SO_4$/0.05% Lubrol, pH7.4, and homogeneous cytochrome b_5 was found in the second protein peak.

A 52–87% yield of apparently homogenously purified cytochrome b_5 was obtained within 8h (see Table 1). Accurate estimation of cytochrome b_5 concentration before solubilization of the microsomal fraction is difficult, because the preparations contain several haemoproteins which interfere with the cytochrome b_5 assay (see Ozols, 1974). Cytochrome b_5 could not be detected in the 105000*g* pellet obtained after solubilization, and thus the yield of cytochrome b_5 in the solubilized microsomal preparation was taken to be 100%.

The absorption spectrum of the purified cytochrome b_5 is shown in Fig. 1, and it indicates that denaturation of the cytochrome b_5 protein has not occurred during purification.

The cytochrome b_5 could be concentrated by addition of an equal volume of cold 90% (w/v) $(NH_4)_2SO_4$ solution, pH7.4, to the purified preparation. After 5min, the mixture was centrifuged at 20000*g* for 15min and the precipitate of cytochrome b_5 floated on top of the $(NH_4)_2SO_4$ solution. The floating cytochrome b_5 could be recovered and redissolved in a small volume of buffer and dialysed overnight. This $(NH_4)_2SO_4$ step allows purified cytochrome b_5 to be obtained at any desired concentration and in any buffer within 24h of the start of the purification.

Biological activity of cytochrome b_5

The biological activity of cytochrome b_5 was determined by assay of 7-ethoxycoumarin *O*-de-ethylation activity in the presence and absence of cytochrome b_5. A reconstituted mono-oxygenase system was constructed by using fraction-I cytochrome *P*-450 from female-rat liver microsomal fractions as described by Kamataki *et al.* (1981). On addition of cytochrome b_5, the *O*-de-ethylation activity of the reconstituted system increased from 0.071 to 0.127nmol/min per nmol of cytochrome *P*-450. This 1.7-fold enhancement of activity by cytochrome is very similar to the 1.6-fold enhancement reported by Kamataki *et al.* (1981).

Purity of cytochrome b_5

Samples of purified microsomal cytochrome b_5 were analysed by electrophoresis in the presence of sodium dodecyl sulphate on 7–16%-polyacrylamide gels (see the Materials and methods section). Only one polypeptide-staining band was visible after gel electrophoresis (Fig. 2). The subunit M_r was determined to be 16600, by comparison of cytochrome b_5 with protein standards of known M_r. This determination was carried out on sodium dodecyl sulphate/11%-polyacrylamide gels (results not shown), as the relationship between $\log M_r$ and mobility was not linear in the 10000–20000-M_r range on the gradient gels.

Discussion

Cytochrome b_5 has been purified to homogeneity in high yield by a rapid simple procedure. The

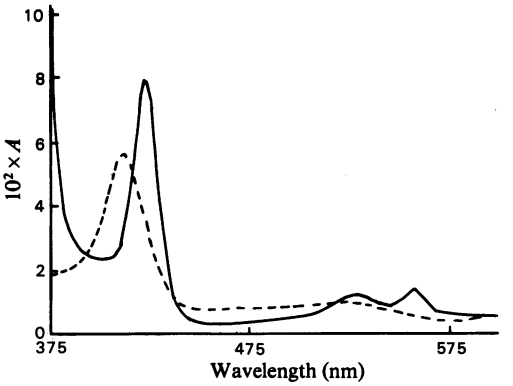

Fig. 1. *Absorption spectrum of rat cytochrome b_5*
Absorption spectra of purified cytochrome b_5 in 0.05M-Tris/acetate buffer, pH8.1, at 25°C: ----, oxidized form of cytochrome b_5; ——, cytochrome b_5 after reduction with solid sodium dithionate.

Table 1. *Purification of rat hepatic microsomal cytochrome b_5*
Data presented are from a single purification experiment. Numbers in parentheses are ranges of values obtained in three separate preparations.

Fraction	Cytochrome b_5 (nmol/mg)	Yield (%)	Purification (fold)
Lubrol-solubilized microsomal fraction	0.63 (0.63–0.65)	100	1
Fractogel TSK DEAE-650(S) eluate	35 (35–43)	65 (52–87)	56 (56–66)

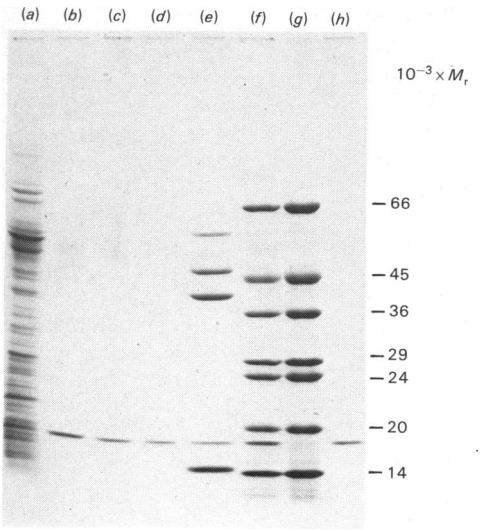

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified cytochrome b_5*

Slab electrophoresis was performed in a 7–16%-acrylamide linear gradient of separating gel (18cm × 8cm) and a 4% stacking gel in the presence of 0.1% sodium dodecyl sulphate (see the Materials and methods section). Tracks: (*a*), rat liver microsomal fraction; (*b*), (*c*), (*d*), purified cytochrome b_5 from three separate preparations; (*e*), purified cytochrome b_5 plus four Boehringer standard proteins; (*f*), purified cytochrome b_5 plus seven Sigma standard proteins; (*g*) standard proteins; (*h*) purified cytochrome b_5 after concentration with $(NH_4)_2SO_4$. The direction of migration is from top to bottom.

number of steps required and the complexity of the method have been greatly decreased by comparison with previously published procedures (see Spatz & Strittmatter, 1971; Ozols, 1974; Chiang, 1981; Kamataki *et al.*, 1981). The properties of the pure enzyme compare favourably with the previous preparations. The subunit M_r obtained, 16600, agrees with the values of 16700 reported by Spatz & Strittmatter (1971) and 16000 reported by Chiang (1981). The specific content of cytochrome b_5 obtained in the purified preparation (35–41nmol/mg) agrees with the values previously reported for rat cytochrome b_5 (Kamataki *et al.*, 1981). The purified cytochrome b_5 exhibited good activity when added to a reconstituted monooxygenase system, indicating that it has not lost its biological activity during purification. Most of the previous publications concerning purification of cytochrome b_5 do not report purification tables or yields of cytochrome b_5. However, Kamataki *et al.* (1981) apparently obtained rat cytochrome b_5 in 17–20% yields. I have also calculated from the data published by Canova-Davis & Waskell (1984) that they purified rabbit cytochrome b_5 in 8.6% yield. The present paper shows that 52–87% yields of rat liver cytochrome b_5 can be obtained in 8h.

In conclusion, I have obtained a preparation of homogeneous cytochrome b_5 in high yield by a much simpler and quicker procedure than has been previously reported.

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