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Hydrodynamic Characterization of Highly Purified and Functionally Active Liver Microsomal Cytochrome P-450†

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ABSTRACT: The subunit molecular weight of highly purified phenobarbital-treated rat liver microsomal cytochrome P-450 (P-450) was estimated to be 55 000 ± 2000 by sedimentation velocity and equilibrium measurements in the presence of sodium dodecyl sulfate. Sedimentation equilibrium studies indicate that, in 0.1 M KCl at its isoelectric pH of 6.5, P-450 undergoes self-association with an apparent weight-average molecular weight (M_w) of about 500 000 at high dilution. The dependence of M_w on concentration could be described by a model in which six to nine P-450 monomers associate with high affinity to form a basic aggregate P_1 which further self-associates isodesmically. Values for k [$\sim 10^{11}$ M^{-1}, where $k = [P_{n+1}]/([P_n][P_1])$; Teller, D. C. (1973) *Methods Enzymol. 27*, 346–411] were found not to change appreciably for P-450 in the presence of the substrate *d*-benzphetamine, phospholipid monomers, or NADPH–cytochrome P-450 reductase. The molecular weight of the P_1 aggregate was decreased by ~100 000 in the presence of phospholipid monomers and increased about twofold in the presence of NADPH–cytochrome P-450 reductase plus phospholipid monomers. The presence of phospholipid micelles raised k about threefold, while 6 M guanidine hydrochloride and 0.1% (w/v) Triton N-101 lowered k to 10^{10} and 10^9 M^{-1}, respectively, and decreased the molecular weight of P_1 to ~150 000. Monomeric P-450 was detected only in the presence of amphipathic detergents. For the isodesmic self-association of the ~400 000-dalton aggregate of P-450 at pH 6.5 and 20 °C, $\Delta G = -14.4 \pm 0.1$ kcal mol^{-1}, $\Delta H = -4.8 \pm 1.3$ kcal mol^{-1}, and $\Delta S = 33 \pm 5$ eu. The results suggest that hydrophobic interaction is important for the isodesmic association of the P_1 aggregate. Several lines of evidence also suggest that P-450 activity is not very dependent upon the state of aggregation of the enzyme. Other P-450's isolated from rats and rabbits were also found to aggregate in a similar manner.

A number of enzymes have been shown to depend upon lipids for activity, as first reviewed by Fleischer & Fleischer (1967) and more recently by Sandermann (1978). While a number of these enzymes have been purified, relatively few detailed studies have been carried out to describe the interactions of such enzymes with lipids and detergents or how these proteins associate and dissociate in solution (McIntyre et al., 1978).

P-450,[1] the terminal oxidase of the microsomal mixed-function oxidase system that metabolizes a variety of endogenous and exogenous chemicals (Lu & West, 1978; Guengerich, 1979a; Coon et al., 1977), is associated with the flavoprotein NADPH–cytochrome P-450 reductase in the endoplasmic reticulum. Both enzymes have been isolated with detergents and purified to apparent homogeneity; a variety of evidence exists for the multiplicity of P-450 in various species (Coon et al., 1976, 1977; Guengerich, 1979a), and three different forms have been purified to apparent homogeneity from rat liver in this laboratory (Guengerich, 1978a).

Rates of P-450-dependent activities of microsomal preparations can be decreased by lipid extraction and restored by the addition of phospholipids (Vore et al., 1974). The activity of the reconstituted enzyme system has been shown to be stimulated by phosphatidylcholine (Strobel et al., 1970). The lipid appears to function at least at two levels. For example, di-12 GPC promotes association of P-450 with its organic substrates and with NADPH–cytochrome P-450 reductase (Guengerich & Coon, 1975; Coon et al., 1976), and these increased affinities are responsible for enhancement of the rate of electron flow (Strobel et al., 1970) and possibly other steps. The presence of phospholipid does not, however, appear to influence the oxidation–reduction potential of P-450 (Guengerich et al., 1975). Previous work suggests that P-450 exists as an aggregate in its active state (Autor et al., 1973), in a complex with NADPH–cytochrome P-450 reductase (Coon et al., 1976; French et al., 1978), but others have suggested that P-450 exists in an active monomeric state (Ingleman-Sundberg, 1977).

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[1] Abbreviations used: P-450, liver microsomal cytochrome P-450; $NaDodSO_4$, sodium dodecyl sulfate; di-12 GPC, L-α-dilauroylglyceryl-3-phosphorylcholine; NaDOC, sodium deoxycholate; EDTA, (ethylenedinitrilo)tetraacetic acid; cmc, critical micelle concentration; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; M_w, weight-average molecular weight; R_S, Stokes' radius; Gdn, guanidine.

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The investigations described herein were initiated to elucidate the catalytic nature of P-450 and its interaction with phospholipids and detergents during isolation and purification. Also, the hydrodynamic properties of P-450 were determined and are compared to those of other proteins for which data have been collected.

Experimental Procedure

Materials. P-450's were purified as previously described (Guengerich 1977, 1978a); the rat "B" fractions discussed in these references were used in this work. These fractions seem to represent homogeneous proteins as judged by electrophoresis in different $NaDodSO_4$–polyacrylamide gel systems and in agarose at eight different pH values, immunological techniques, N-terminal analysis, specific contents of P-450 per milligram of protein, linearity of 7-ethoxycoumarin *O*-deethylase kinetics (Guengerich, 1978a), and the hydrodynamic studies described in this report. NADPH–cytochrome P-450 reductase was purified to apparent homogeneity as described elsewhere (Guengerich, 1978b), except that FMN was excluded from buffers; the specific activity toward cytochrome *c* was 67 μmol min^{-1} mg^{-1} and the A_{455}/A_{380} ratio was 1.15. Guanidine hydrochloride was purchased from Heico and $NaDodSO_4$ was from Bio-Rad. [^{35}S]$NaDodSO_4$ was obtained from New England Nuclear. Cholic acid (Eastman) was recrystallized twice as previously described (Guengerich, 1978a). Emulgens 911 and 913 were gifts of Kao-Atlas (Japan); *d*-benzphetamine hydrochloride and *d*-benzylamphetamine hydrochloride were gifts of Dr. P. W. O'Connell of Upjohn (Kalamazoo, MI).

In the synthesis of [*N-methyl*-^{14}C]benzphetamine, a mixture of 1 mmol of benzylamphetamine (free base), 1.1 mmol of $^{14}CH_3I$ (2 mCi), and 1.1 mmol of K_2CO_3 was stirred overnight in 30 mL of acetone. Acetone was removed in vacuo and the product was extracted 3 times from 0.1 N NaOH into $CHCl_3$. The combined $CHCl_3$ layers were dried over anhydrous $MgSO_4$, saturated with dry HCl gas, and concentrated to dryness. Crystallization of the residue from ethyl acetate gave 0.5 mmol of [*N-methyl*-^{14}C]benzphetamine hydrochloride [mp 194–195 °C (uncor)];[2] the NMR spectrum was identical with that of an authentic sample (in $CDCl_3$); the M^+ of the free base was at *m/e* 225.

Preparation of P-450 for Hydrodynamic Studies. For the studies in the absence of $NaDodSO_4$ or guanidine hydrochloride, P-450 was dialyzed overnight against 200 volumes of 50 mM potassium phosphate buffer (pH 6.5) containing 100 mM KCl, 0.1 mM EDTA, and 0.02% NaN_3. After such treatment, P-450 was stable for at least 5 days (i.e., less than 10% of the spectrally detectable P-450 was lost), even in the presence of 0.05 mM di-12 GPC or 0.1% (w/v) Emulgen 913 at either 25 or 4 °C.

For the studies carried out in the presence of $NaDodSO_4$, P-450 was first dialyzed against 200 volumes of water and then $NaDodSO_4$ (0.2% w/v) to which dithiothreitol (10 mM) and Tris-HCl (0.5 M, pH 8.5) were added. After 30 min, iodoacetate was added to 40 mM and carboxymethylation proceeded in the dark for 2 h at 25 °C. The mixture was dialyzed against three changes of 0.1 M sodium phosphate buffer (pH 8.0, 200 volumes) over 2 days and then against water. Apo-P-450 was prepared by acetone–HCl treatment (Guengerich et al., 1975); the pellet was washed 3 times with CH_3OH, dried under N_2, and solubilized in 10 mM Tris–acetate buffer (pH 7.4) containing 0.2% $NaDodSO_4$. The procedure for heme removal was repeated twice, and the residue was solubilized in 50 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, and 0.2% $NaDodSO_4$ and filtered through glass wool.

The carboxymethylation procedure was identical for the guanidine hydrochloride experiment, except that 6 M guanidine hydrochloride replaced $NaDodSO_4$; the heme extraction procedure was not used.

Other Assays. $NaDodSO_4$ binding to P-450 analysis was carried out by using 0.9 × 50 cm silicon-coated glass columns filled with Bio-Gel P-10 (Bio-Rad) and the basic procedure of Hummel & Dreyer (1962). Radioactivity troughs were used for calculations; stock [^{35}S]$NaDodSO_4$ was diluted to give approximately 10^3 dpm/mg of $NaDodSO_4$. cmc's were estimated by using Rhodamine 6G as described (Becher, 1962; Bonsen et al., 1972). The level of Emulgen 913 bound to a purified P-450 preparation was estimated by the technique of Garewal (1973) as modified by Goldstein & Blecher (1975). Because isoelectric focusing of P-450's is accompanied by certain artifacts (Guengerich, 1979b), the isoelectric points of P-450's were estimated by carrying out electrophoresis in agarose (1.5% w/v; Bio-Rad Ultrapure) using 0.1 M Tris-HCl–0.1 M glycine buffers at eight different pH values over the range of 4–8 and plotting the P-450 mobility (relative to bromphenol blue) as a function of pH. The following p*I* values were estimated: phenobarbital-treated rat P-450, 6.5; 3-methylcholanthrene-treated rat P-450, 5.6; phenobarbital-treated rabbit P-450 LM_2, 8.1; β-naphthoflavone-treated rabbit LM_4, 7.0 (Guengerich, 1979a).

Gel filtration was carried out by using 0.9 × 50 cm silicone-coated glass columns filled with Sepharose CL-4B (Pharmacia), and individual fractions (≤0.5 g) were weighed in all cases. Standards for the Sepharose 4B columns used for R_S determinations were bovine serum albumin, ovalbumin, and chymotrypsinogen A; R_S values used were as determined by Tanford et al. (1974). Molecular weight standards used for calibration of the Sephadex G-200 gel filtration column used for the Emulgen 911–cholate experiment were bovine liver catalase (244 000), fluoresceinisothiocyanate-labeled bovine serum albumin (68 000), cytochrome *c* (12 800), and $K_3Fe_3(CN)_6$.

Benzphetamine demethylation was determined as described (Guengerich et al., 1975). Protein was estimated as described by Lowry et al. (1951) and P-450 was determined according to Omura & Sato (1964) using a Cary 219 spectrophotometer in the automatic base-line correction mode at 20 ± 1 °C.

Measurement of Diffusion Constants. Diffusion constants were determined by using the free diffusion method (Gosting, 1956; McIntyre et al., 1978) with the following changes. Each tube contained 50 mM potassium phosphate buffer (nominal pH 6.5 as measured with a hydrogen electrode), 100 mM KCl, 0.1 mM EDTA, 0.02% NaN_3, 0.05 mM di-12 GPC, and 93.8% (v/v) D_2O; the lower half also contained 5% (w/v) sucrose (Schwarz/Mann Ultrapure), 1.0 μM P-450, and 1.0 μM NADPH–cytochrome P-450 reductase. The solvent density (1.100 g/mL) was adjusted with D_2O (Reynolds & Tanford, 1976) to offset the partial specific volume of the di-12 GPC ($\bar{\nu}_D$ = 0.909 cm^3 g^{-1}), which was calculated by extrapolation of previous data (Tausk et al., 1974a,b). The tube size was 5.0 mL (area = 1.13 cm^2), the time was 63 h, and the temperature was 20 ± 0.5 °C. The contents of each tube were removed by using a Buchler Auto Densiflow device coupled to a peristaltic pump. The effluent passed through the flow

[2] The melting point listed in the Merck Index (1968) is 129–130 °C and the melting point of a sample obtained from Upjohn was 152–154 °C (the melting point of benzylamphetamine hydrochloride was 175–176 °C). The spectral data clearly indicate that the product was benzphetamine.

cell of a Varian Varichorm flow cell with the wavelength set at 415 nm (for determination of P-450), and 0.11-mL fractions were collected, weighed, and assayed for NADPH–cytochrome P-450 reductase (Guengerich, 1978b). Three tubes were assayed at $t = 0$ as controls; the resulting curves were averaged and subtracted from the experimental ($t = 63$ h) curves prior to analysis of the data. Diffusion constants were estimated by using a nonlinear least-squares fit of the data to the integrated diffusion equation (Oswald & Freeman, 1979). Diffusion constants were not corrected to $D_{20,w}$.

Sedimentation Velocity Experiments. Sedimentation velocity measurements were made by using a Beckman Model E ultracentrifuge with scanner optics. For sedimentation velocity studies in the absence of detergent, the sedimentation coefficients were calculated by using second-moment analysis (Schachman, 1959). The sedimentation coefficient of the P-450–$NaDodSO_4$ complex was determined for P-450 samples in 0.2% $NaDodSO_4$ and 50 mM sodium phosphate buffer (pH 6.5) at 20 °C by using a rotor speed of 56 000 with double-sector 12-mm Kel F centerpieces. The boundary movement was monitored by using scanner optics, and the sedimentation coefficient was calculated by using the half-height of the boundary. For the P-450–$NaDodSO_4$ complex, sedimentation coefficients were corrected to water at 20 °C by using an effective specific volume calculated from the protein partial specific volume and the grams of bound detergent per gram of protein. Protein partial specific volumes were calculated (Cohn & Edsall, 1943) from amino acid compositions (Guengerich, 1978a). R_S values were determined by gel filtration (Tanford et al., 1974). For the reconstituted P-450 reductase complex, molecular weights were estimated from the expression

$$M_{s/D} = \frac{RT}{1 - \bar{\nu}_2\rho_0}\frac{s}{D}$$

where $M_{s/D}$ is the apparent molecular weight, T is the absolute temperature, $\bar{\nu}_2$ is the protein partial specific volume, ρ_0 is the solvent density, s is the sedimentation coefficient, and D is the diffusion constant. Values for s and D were determined in the same solvent and were not corrected to 20 °C and water.

The sedimentation coefficient of active P-450 in the reconstituted system containing NADPH–cytochrome P-450 reductase was determined by using the active enzyme sedimentation method of Cohen & Mire (1971) as modified by McIntyre et al. (1978). A Vinograd-type double-sector centerpiece (Beckman No. 331359) was used in an AN-H rotor with the Model E ultracentrifuge. An enzyme solution (10 μL) corresponding to the lower portion of the solution used for the diffusion constant determination (without sucrose) was placed in the sample well in the centerpiece of the cell, and the rotor was equilibrated at 20 °C inside the centrifuge. As the rotor accelerated to 52 000 rpm, the active enzyme complex layered onto the surface of an assay solution in the main cell compartment containing 0.15 mM NADPH and all components in the sample compartment solution except the enzymes. The sedimentation of the enzyme complex was monitored by the disappearance of NADPH (ΔA_{340}). The enzyme was placed in the well corresponding to the solvent (left-hand side) side of the centerpiece so that the loss of NADPH would be recorded as a relative increase in optical density in the solution sector. The sedimentation coefficient was estimated by the difference method of Cohen & Mire (1971). During the run, a parallel incubation (with 0.15 mM NADPH, 1.0 μM P-450, and 1.0 μM NADPH–cytochrome P-450 reductase) was carried out at 20 ± 1 °C in a Cary 219 spectrophotometer.

Sedimentation Equilibrium Experiments. Sedimentation equilibrium studies were carried out in the Beckman Model E ultracentrifuge in double-sector cells using both low-speed techniques (Van Holde & Baldwin, 1958) and the meniscus depletion method of Yphantis (1964). One sector of each cell contained solvent; the other sector contained a 2.8-mm column of the protein sample which had been previously equilibrated by dialysis with the solvent. Equilibrium was attained at speeds of 4000–18 000 rpm (10 000–26 000 in the presence of detergent). In every case, the true cell base line was determined following overspeeding after equilibrium scans were made at the indicated rotor speeds. A_{415} or A_{280} scans were made at equilibrium, and the point-weight-average molecular weight was calculated (Holladay et al., 1977) from the relationship

$$M_{w_{r,app}} = \frac{2RT}{(1 - \bar{\nu}_2\rho)\omega^2}\frac{\mathrm{d}\ln C}{\mathrm{d}r^2}$$

where R is the universal gas constant, T is the absolute temperature, ω is the angular velocity, and $\mathrm{d}\ln C/\mathrm{d}r^2$ is the slope of the line of the natural logarithm of concentration vs. the square of the radial distance from the axis of rotation. For all sedimentation equilibrium experiments the $\ln C$ vs. r^2 data were fitted to the equation $\ln C = \alpha + \alpha_1 r^2 + \alpha_2 r^4$, and the linearity of the plot was determined by calculation of the Student's t statistic ($\alpha_2/\sigma_{\alpha_2}$) for the coefficient α_2. The z-average molecular weight (M_z) was calculated pointwise as previously described (Holladay et al., 1977). D_2O was added to solutions to bring the product $\bar{\nu}_D\rho$ to unity for added detergents and lipids to negate the contribution of such materials to the estimated molecular weight (Reynolds & Tanford, 1976); the values of $\bar{\nu}_D$ for di-12 GPC used were 0.909 cm^3 g^{-1} at 0.05 mM and 0.973 cm^3 g^{-1} at 0.75 mM concentrations [extrapolated from data of Tausk et al. (1974a,b)]; a $\bar{\nu}_D$ of 0.922 cm^3 g^{-1} (Tanford et al., 1974) was used for Triton N-101, a nonylphenyl ether quite similar to the detergents Emulgen 911, Emulgen 913, and Renex 690 which are often used in P-450 purification (Guengerich, 1977, 1978a, 1979a).

Results

Subunit Molecular Weight Studies. Previous studies with six different polyacrylamide gel electrophoretic systems indicated that the subunit molecular weights of phenobarbital- and 3-methylcholanthrene-treated rat P-450 preparations are approximately 55 000 (Guengerich, 1978a). Values of 47 000–60 000 have been obtained for various P-450's isolated from rat, rabbit, mouse, and human microsomes (Guengerich, 1979a). A number of proteins bind unusual amounts of $NaDodSO_4$ and give rise to spurious molecular weight estimates using $NaDodSO_4$ gel electrophoresis (Segrest et al., 1971; Rubin & Tzagoloff, 1973). Plots of log (relative mobility) vs. acrylamide concentration (Ferguson, 1964; Segrest et al., 1971) suggested that the observed molecular weights are reasonable estimates (Guengerich, 1978a). Preliminary experiments indicated that P-450 is not completely dissociated in the presence of 6 M Gdn·HCl, even after reduction and carboxymethylation (Guengerich, 1978a). Studies were attempted by using hexadecyltrimethylammonium bromide, which has a partial specific volume of unity (Tanford et al., 1974); however, this cationic detergent precipitated from solution at detergent concentrations slightly above the cmc at 20 °C. For these reasons, hydrodynamic characterization of P-450 monomers was done in $NaDodSO_4$ solutions.

Phenobarbital-treated rat P-450 was found to have subunit molecular weights of 57 000 and 53 600 as determined by sedimentation velocity and equilibrium measurements, respectively (Table I). 3-Methylcholanthrene-treated rat P-450

Table I: Determination of P-450 Monomeric Molecular Weight in the Presence of $NaDodSO_4$

method	subunit molecular weight: phenobarbital rat P-450	subunit molecular weight: 3-methylcholanthrene rat P-450
polyacrylamide gel electrophoresis[a]	45 900–53 100	49 900–56 950
sedimentation velocity and R_S (gel filtration)	57 000[b]	55 800[c]
sedimentation equilibrium	53 600 (±1100)[d]	[e]

[a] Guengerich (1978a). [b] Calculated by using $s_{20,w}$ = 3.19 S, R_S = 77 Å, $\bar{v}$ = 0.742 $cm^3\ g^{-1}$, and 1.7 g of $NaDodSO_4$ bound per g of protein (Reynolds & Tanford, 1976). [c] Calculated by using $s_{20,w}$ = 2.99 S, R_S = 76 Å $\bar{v}$ = 0.741 $cm^3\ g^{-1}$, and 1.6 g of $NaDodSO_4$ bound per g of protein (Reynolds & Tanford, 1976). [d] Conditions: 20 °C; 26 000 rpm. [e] Not determined.

was determined to have a subunit molecular weight of 55 800 by using sedimentation velocity measurements. Both proteins behaved as homogeneous species during these measurements.

In the sedimentation equilibrium studies, the thorough extraction of residual heme was found to be extremely important. A_{280} data were obscured by small molecular weight impurities attributed to heme (not bound in its native state) or products formed during carboxymethylation of the preparations when as little as 5–10% of the residual heme was present.

Catalytic Activity of P-450 in the Presence of Phospholipid and Detergent. P-450 exhibits monooxygenase activity toward a wide variety of hydrophobic substrates; *d*-benzphetamine *N*-demethylase activity was examined in these studies because of the solubility of the substrate, the sensitivity of the ^{14}C extraction assay, and the extensive use of this activity in other P-450 studies (Coon et al., 1976; Lu & West, 1978). All studies were carried out at 20 °C in 50 mM potassium phosphate buffer (pH 6.5; i.e., p*I* of this P-450) containing 100 mM KCl, 0.1 mM EDTA, and 0.02% NaN_3 to facilitate comparisons with hydrodynamic data, although the benzphetamine demethylase activity was markedly lower than that at pH 7.7 and 30 °C (Guengerich, 1977).

The P-450 preparation (phenobarbital-treated rat) used in these studies was found to contain 29 μg of residual Emulgen 913 bound per mg of P-450 (about 2 nmol of average monomer detergent per nmol of P-450). The procedures used for the preparation of P-450 have previously been shown to reduce the levels of bound phospholipid and cholate to very low levels (<1 nmol/nmol of P-450) (Guengerich & Coon, 1975; Autor et al., 1973). The cmc's determined in this buffer system were 0.078 mM for di-12 GPC, 0.65 mM for NaDOC, and 27 μg mL^{-1} for Triton N-101; the cmc for di-12 GPC was 0.094 mM in the presence of 0.25 mM NaDOC.

The data presented in Figure 1 indicate that the reductase-coupled P-450 system is stimulated by di-12 GPC; optimal stimulation was observed at a concentration of about 0.05 mM di-12 GPC when the P-450 concentration was 2.8 μM. Di-12 GPC stimulated P-450 activity has previously been demonstrated to be enhanced in the presence of 0.25 mM NaDOC (Haugen et al., 1975; Lu & West, 1978); such a phenomenon was also observed here. Above the apparent cmc, activity remained constant to di-12 GPC concentrations of up to 0.75 mM di-12 GPC. Substitution of 0.1% (w/v) Triton N-101 for 0.05 mM di-12 GPC and 0.25 mM NaDOC gave a nearly identical rate of demethylation (1.48 vs. 1.44 min^{-1}, respectively). In experiments carried out with 50 nM P-450 concentrations, optimal di-12 GPC stimulation was also observed at 0.04 to 0.05 mM concentrations, in line with previous work

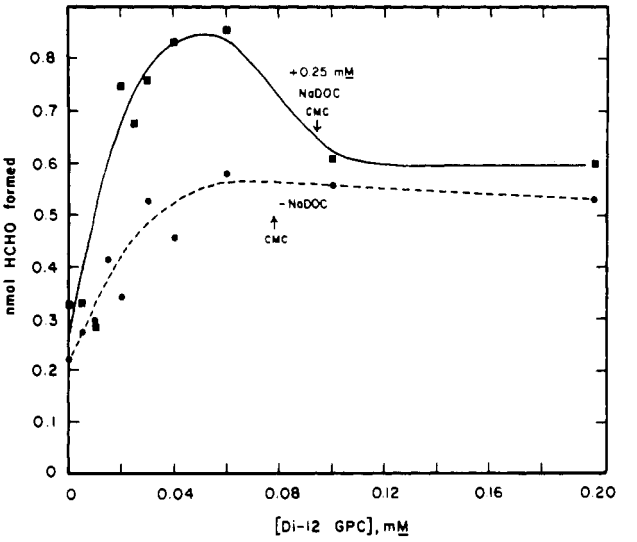

FIGURE 1: Dependence of benzphetamine demethylation of P-450 upon di-12 GPC in the presence of NADPH and NADPH–cytochrome P-450 reductase. Incubations contained P-450 (2.8 μM), NADPH–cytochrome P-450 reductase (2.9 μM), potassium phosphate (50 mM, pH 6.5), KCl (100 mM), EDTA (0.1 mM), [*N-methyl*-^{14}C]benzphetamine (1 mM), NaDOC (0.25 mM, when noted), and the indicated concentration of di-12 GPC in a final volume of 75 μL. Reaction was initiated by the addition of NADPH to 0.4 mM and stopped after 5 min at 20 °C by the addition of 1.5 mL of 0.1 N NaOH. Residual substrate was extracted from $H^{14}CHO$ 3 times with $CHCl_3$ (Guengerich et al., 1975); 0.5-mL aliquots of the aqueous phases were neutralized by the addition of 0.5 mL of 0.1 N HCl–0.2 M Tris–acetate (pH 7.4) and counted in 5 mL of Amersham/Searle ACS cocktail. Control experiments contained all components except NADPH.

(Guengerich, 1977; Lu & West, 1978; Lu et al., 1974; Coon et al., 1976).

Activity was also examined as a function of P-450 concentration (Figure 2). The rate of NADPH oxidation was nearly constant over the range of 15 nM–4 μM. The rate of benzphetamine N-demethylation appeared to peak near 0.12 μM but did not vary more than twofold over the range of concentrations used in the hydrodynamic studies.

Sedimentation Equilibrium Studies with P-450 in the Absence of Amphipathic Detergents. Because P-450 is not active under the detergent conditions used to establish the monomeric molecular weight, the hydrodynamic properties of the enzyme were studied in the absence of $NaDodSO_4$. All studies were carried out with the phenobarbital-treated rat P-450 preparation at its p*I*, pH 6.5, in the presence of 50 mM potassium phosphate and 100 mM KCl to minimize charge interactions.

Analysis of the meniscus depletion (high-speed) data suggested the presence of an aggregate of about 500 000 daltons whose size was not affected substantially by the presence of benzphetamine or 0.05 mM di-12 GPC: the analysis of the ln C_r vs. r^2 data showed that P-450 formed a large aggregate even at the lowest detectable concentrations (50 nM) (Table II). Under these conditions the aggregate appeared to distribute as a single species. In the presence of 0.75 mM di-12 GPC or the reductase, the size of this aggregate was significantly higher. The molecular weights obtained in the presence of detergents and lipids represent the molecular weight of the protein portion of the lipid–P-450 complex since the solvent density was adjusted with D_2O to negate the lipid contribution to the molecular weight of the complex. The addition of 6 M Gdn·HCl gave an aggregate of smaller size and a *t* statistic not consistent with homogeneity. The addition of 0.1% Triton N-101 also decreased the apparent molecular weight but failed to dissociate P-450 into monomers.

The low-speed data for P-450 were not consistent with ho-

Table II: Sedimentation Equilibrium Data for Phenobarbital-Treated Rat P-450 Systems[a]

expt	system	high-speed data		low-speed data: isodesmic model[b]		low-speed data: two-component model[c]		
		$M_w \pm$ SE	t	$M_1 \pm$ SE	$k \pm$ SE $\times 10^{-10}$ (M^{-1})	M_1	M_2	% $SE_{M_1M_2}$
1	P-450: A_{415}[d]	514 000 ± 3900	−1.61	325 000 ± 20 800	13.2 ± 1.5	456 600	1 659 000	8
2	P-450: A_{280}[e]	537 000 ± 11 200	−0.51	495 200 ± 31 500	7.3 ± 0.9	480 600	1 555 000	14
	P-450: A_{415}[e]	614 000 ± 24 800	−3.06	531 600 ± 33 100	8.9 ± 1.0	660 000	2 401 000	17
3	P-450, benzphetamine: A_{415} data[e]	640 000 ± 13 900	−2.88	406 000 ± 68 700	17.4 ± 5.1	658 400	2 533 000	20
4	P-450, benzphetamine, 0.05 mM di-12 GPC: A_{415}[f]	606 000 ± 10 000	0.96	226 300 ± 77 900	15.4 ± 9.2	559 200	2 248 000	9
5	P-450, benzphetamine, 0.75 mM di-12 GPC: A_{415}[f]	1 115 000 ± 15 000	−2.19	446 800 ± 73 800	38.5 ± 11.0	875 000	3 501 000	14
6	P-450, reductase, benzphetamine, 0.05 mM di-12 GPC: A_{415}[f]	803 000 ± 20 800	−1.04	618 600 ± 64 500	9.0 ± 1.7	706 200	2 448 000	16
	P-450, reductase, benzphetamine, 0.05 mM di-12 GPC: A_{470}[f]	738 000 ± 15 200	1.02	702 200 ± 77 500	8.3 ± 1.7	568 000	2 304 000	19
7	P-450, benzphetamine, 0.1% Triton N-101:[g] A_{415}	145 000 ± 3700	−6.15	134 800 ± 7200	0.08 ± 0.099	31 700	131 800	210
8	P-450, 6 M Gdn·HCl: A_{280}[h]	204 000 ± 3600	10.0	178 500 ± 9900	1.06 ± 0.17	145 900	359 100	17

[a] All experiments were carried out at 20 °C. Low-speed data were obtained at the indicated velocities by using the technique of Van Holde & Baldwin (1958). High-speed data were obtained at the indicated velocities by using the method of Yphantis (1964). The weight-average molecular weight (M_w) and t statistic were determined from the high-speed data as described under Experimental Procedure. Low-speed data were fitted to two models. The isodesmic model (Teller, 1973) yields an extrapolated value of M_1 for P_1 (the smallest detectable entity), and $k = [P_{n+1}]/([P_n][P_1])$. The ideal two-component model (Sophianopolous & Van Holde, 1964) yields molecular weights M_1 and M_2, which are assumed to be the only components present. [b] $M_w^2 = M_1^2 + 4KM_1^2C$ (Teller, 1973); $k = KM_1$; $SE_{M_1} = (\sigma^2{}_{M_1}/3M_1^2)^{1/2}$; $SE_k = K[\sigma^2{}_4KM_1^2/16K^2M_1^4 + \sigma^2{}_{M_1}{}^2/M_1^4]^{1/2}$; $SE_K = M_1 SE_k$. [c] $M_z = (M_1 + M_2) - M_1M_2M_w^{-1}$ (Sophianopolous & Van Holde, 1964). % $SE_{M_1M_2} = SE_{M_1M_2}/M_1M_2$. [d] Data from single experiment; low-speed was 4800 rpm; high speed was 8000 rpm. [e] Low speed was 4000 rpm; high speed was 7200 rpm. [f] Low speed was 3600 rpm; high speed was 7200 rpm. [g] Low speed was 7200 rpm; high speed was 18 000 rpm. [h] Single speed was 15 000 rpm.

mogeneity. Analysis using an ideal two-component model (Sophianopoulous & Van Holde, 1964) gave molecular weight values for the smaller component that were, in general, slightly less than those found from the analysis of the high-speed data. However, plots of M_z vs. $M_{w_r}^{-1}$ (an ideal two-component model) were not linear. The M_{w_r} vs. concentration data were found to give good fits by using an ideal indefinitely associating (isodesmic) model plotted as $M_{w_r}^2$ vs. P-450 concentration (Teller, 1973) (Figure 3). In the presence of Triton N-101 or Gdn·HCl, the smallest observed species (P_1) had a molecular weight corresponding to a P-450 trimer (Table II). In all other cases, the molecular weight of P_1 ranged between 200 000 and 700 000. This model yields values for k, the association constant, for the combination of the basic aggregate P_1 (having a molecular weight of M_1) with aggregate P_n to form P_{n+1}; i.e., $k = [P_{n+1}]/([P_n][P_1])$. The determined values of k were found to be quite similar under most conditions (i.e., about 10^{11} M^{-1}). The k values also indicated that P-450 shows a somewhat greater tendency to aggregate (with a similar M_1) in the presence of a high concentration of di-12 GPC. P-450 showed much less tendency to aggregate in the presence of Gdn·HCl or Triton N-101.

Good agreement between A_{280} and A_{415} data was obtained for P-450 (in the absence of added substrate and lipid). In the experiment containing reductase, 90% of the A_{415} was due to P-450 and about 65% of the A_{470} was due to the flavoprotein reductase; the similarity of the molecular weight distributions calculated at the two wavelengths is consistent with a complex of the two proteins being formed.

The sedimentation equilibrium experiment with P-450 alone was repeated, varying the temperature over the range 10–26 °C (Table III). As the temperature was increased, the molecular weight of P_1 became larger and k became smaller. The data of Table III were used to calculate thermodynamic parameters for the association of P-450 in the isodesmic model: $\Delta G_{20°C} = -14.4 \pm 0.1$ kcal mol^{-1}, $\Delta H = -4.8 \pm 1.3$ kcal mol^{-1}, and $\Delta S = 33 \pm 5$ eu. Two other experiments at 20 °C yielded estimates for $\Delta G_{20°C}$ of −14.9 and −14.6 kcal mol^{-1}.

Table III: Variation of Apparent M_1 and Isodesmic Association Constant of Cytochrome P-450 as a Function of Temperature[a]

T (°C)	$M_{w_{cell,volume}}$[b]	M_1 (isodesmic model)	$k \times 10^{-10}$ (M^{-1})
10	480 400	235 700	8.2
14	516 500	308 800	5.94
18	515 600	306 300	6.16
22	528 700	355 200	4.96
26	547 400	372 900	5.09

[a] The rotor speed was 4400 rpm, the buffer contained 0.1 M KCl, 50 mM potassium phosphate (pH 6.5), 0.1 mM EDTA, and 0.02% NaN_3, and the temperature was raised from 10 to 26 °C after equilibrium was reached at each indicated temperature. [b] Calculated from the equation $M_{w_{cell,volume}} = (1/A)[1/(b^2 - a^2)] \ln (C_b/C_a)$ where $A = (1 - \bar{v}_2\rho)\omega^2/(2RT)$.

Table IV: Sedimentation Equilibrium Data for Other P-450's Fitted to an Ideal Isodesmic Model[a]

enzyme	M_1	$k \pm$ SE $\times 10^{-10}$ (M^{-1})	$\Delta G_{20°C}$ (kcal mol^{-1})
3-methylcholanthrene-treated rat phenobarbital-treated rabbit P-450 (LM-2)	408 000 ± 36 700	8.9 ± 1.8	−14.7
β-naphthoflavone-treated rabbit P-450 (LM-4)	390 000 ± 19 000	7.8 ± 1.6	−14.6
	422 000 ± 21 000	3.2 ± 1.1	−13.6

[a] Data were obtained and computed by using the general procedures referred to under Table II and under Experimental Procedure.

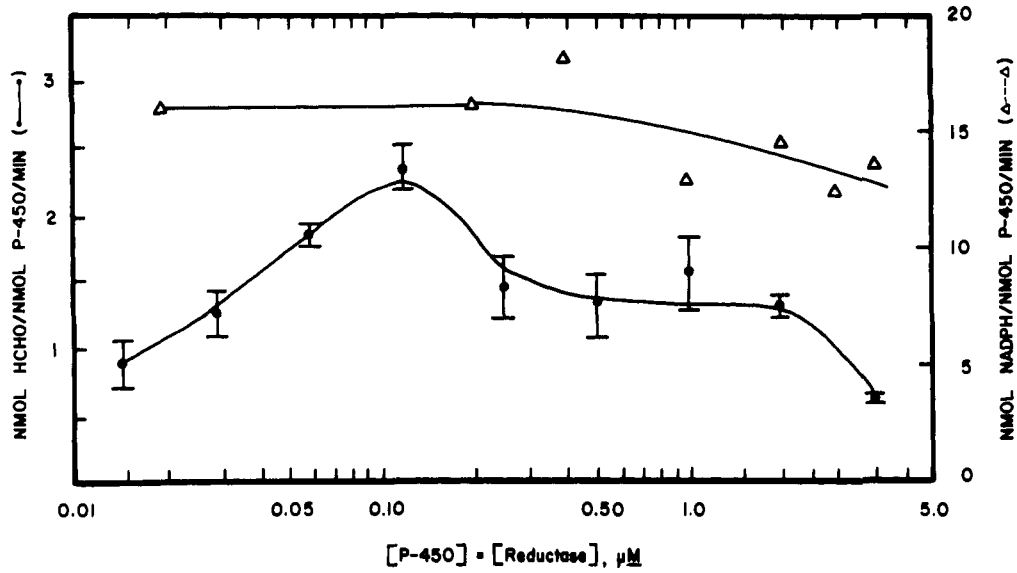

FIGURE 2: P-450 activities as a function of protein concentration in reconstituted enzyme systems. Equal concentrations of phenobarbital-treated rat P-450 and NADPH–cytochrome P-450 reductase were incubated in the presence of 50 mM potassium phosphate (pH 6.5), 0.1 M KCl, 0.02% NaN_3, 0.1 mM EDTA, 0.05 mM di-12 GPC, 0.15 mM NADPH, and 1.0 mM [*N-methyl*-^{14}C]benzphetamine hydrochloride at 20 °C. NADPH oxidation (□) was measured at 340 nm in a Cary 219 spectrophotometer, and production of $H^{14}CHO$ (●) (duplicate experiments ± SD) was assayed as described (Guengerich et al., 1975). All incubations were carried out for time intervals in which NADPH oxidation followed zero-order kinetics.

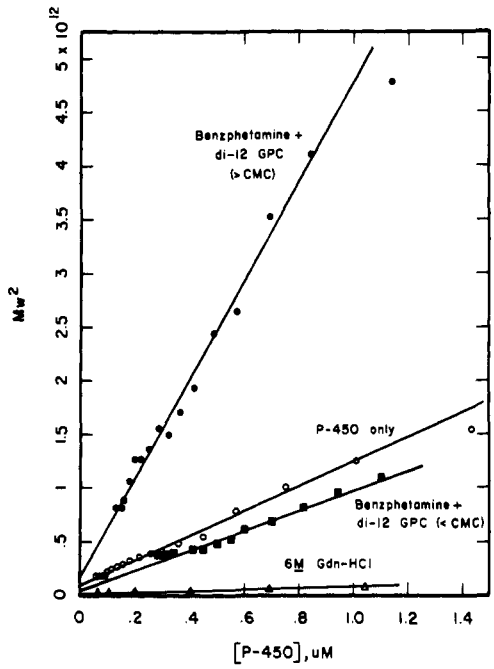

FIGURE 3: Self-association of P-450 plotted to fit the isodesmic model. Data from Table II were plotted as the square of the weight-average molecular weight ($M_{w,app}^2$) vs. P-450 concentration: experiment 5 (●); experiment 2 (○); experiment 4 (■); experiment 8 (△).

Other microsomal P-450's also appear to behave in a manner similar to that of the phenobarbital-treated rat preparation. Three other apparently homogeneous liver P-450 preparations were submitted to sedimentation equilibrium studies in the absence of detergents and lipids, at their respective p*I* values,[3] in 50 mM potassium phosphate buffer containing 100 mM KCl, 0.1 mM EDTA, and 0.02% NaN_3; the resulting data were plotted to fit the isodesmic model, as shown in Table IV.

Sedimentation Velocity Experiments with the Reconstituted P-450/NADPH–Cytochrome P-450 Reductase System. Sedimentation velocity measurements were carried out at 20 °C in 50 mM potassium phosphate, 100 mM KCl, 0.1 mM EDTA, 0.02% NaN_3, 0.05 mM di-12 GPC, and sufficient D_2O (93.8% v/v) to offset the contribution of di-12 GPC to the molecular weight. Phenobarbital-treated rat liver P-450 (at 1.0 μM) sedimented at 12.9 ± 0.8 s and NADPH–cytochrome P-450 reductase (1.0 μM) sedimented at 11.3 ± 0.4 s in separate experiments. When the two proteins were mixed (1.0 μM concentration of each), the observed sedimentation coefficient was 14.7 ± 1.2 s for the P-450 (based upon A_{415} measurements); diffusion constants of (1.36 ± 0.09) × 10^{-7} and (1.35 ± 0.07) × 10^{-7} cm^2 s^{-1} were established for P-450 and the reductase, respectively, in the mixture of the two proteins. These values yielded an estimate for $M_{s/D}$ of 1.4 (±0.3) × 10^6 for the P-450–reductase complex in the active reconstituted enzyme system. In a similar low-speed equilibrium experiment (Table II, experiment 6), the weight-average molecular weight increased from 1.28 × 10^6 to 1.6 × 10^6 over the P-450 concentration range of 0.5–1.0 μM. For an isodesmic system it is likely that $M_{s/D}$ will be somewhat less than M_w but greater than M_n (Jullander, 1946). The small difference between $M_{s/D}$ and M_w for the reconstituted enzyme system at 1 μM concentration is well within the limits of experimental error for $M_{s/D}$ and the expected theoretical difference between $M_{s/D}$ and M_w.

The same mixture was used in an active enzyme sedimentation velocity experiment, where the oxidation of NADPH was measured as a function of time and radial distance. The activity was found to sediment in the same manner as that of the P-450 and reductase, with a value of 10.7 ± 0.6 s. The leading edge of the activity was quite broad and closely paralleled the P-450 profile, producing a fairly constant NADPH oxidation/P-450 ratio over the range of apparent molecular weight. The higher sedimentation coefficient determined by A_{415} measurements could result if the very high molecular weight species were less enzymatically active than the lower molecular weight species. High activity was present at the top of the column during the start of the experiment, but the total activity decreased as the protein sedimented. In a control experiment carried out in a conventional spectrophotometer,

[3] The pH was 6.5 in the case of 3-methylcholanthrene-treated rat P-450 (cf. p*I* of 5.6).

activity followed zero-order kinetics for at least 15 min.

Discussion

The molecular weight of the P-450 subunit obtained by hydrodynamic measurements is about 55 000, in good agreement with estimates found by $NaDodSO_4$–polyacrylamide gel electrophoresis. Thus, purified rat liver P-450's should contain about 18 nmol of spectrally detectable P-450 per mg of protein, if no heme has been lost. Such is the case for the preparations considered here (Guengerich, 1978a).

The sedimentation equilibrium results are consistent with the existence of a complex of P-450 and NADPH–cytochrome P-450 reductase (Coon et al., 1976), since a mixture of the two proteins gave a molecular weight for P_1 (isodesmic model) roughly twice as high as that for P-450 alone (Table II). Moreover, the A_{415} data (primarily due to P-450) and the A_{470} data (primarily due to reductase) yielded almost superimposable $M_{w,app}$ vs. concentration relationships. Using subunit molecular weights of 55 000 for P-450 and 74 000 for the reductase (Guengerich, 1978b; Knapp et al., 1977), one can estimate that four to six monomers of each enzyme are present in such a complex. The free energy involved in the formation of such a complex would appear to be similar to that involved in the self-association of P-450.

The data indicate that P-450 resists dissociation into monomers except in the presence of strong amphipathic detergents. While Gdn·HCl and nonionic detergent lower the free energy of association, the smallest detectable aggregate in each case appeared to correspond to about a trimer (Table II). Two other groups have reported that P-450 exists as a monomer in the presence of nonionic detergents. Warner et al. (1978) carried out gel filtration studies in the presence of the detergents Emulgen 911 and cholate; P-450 migrated as a broad species with a monomeric molecular weight as judged by one set of standards and with a dimeric molecular weight as judged by another set of standards. We have tried to repeat these results in this laboratory, but three individual experiments all yielded apparent molecular weights of 250 000–300 000, based upon the standards described under Experimental Procedure. Ingelman-Sundberg (1977) carried out sucrose density experiments with a rabbit liver P-450 preparation (LM_2) in the presence of Triton X-100 and concluded that the monomer bound an equal weight of the detergent and is the catalytically active species. However, detergent binding was assumed to be independent of the sucrose concentration, which may not be the case (Tanford et al., 1974), and no mention was made as to whether the s and R_S values used for the standards were appropriate for detergent solutions. Moreover, the experimental design did not permit the parameter ϕ_2 to be obtained with sufficient accuracy.

It is instructive to compare the thermodynamics of the self-association of P-450 with those of water-soluble proteins.[4] The dimerization of lysozyme at pH 8 yields estimates of $\Delta G_{20°C} = -3.4$ kcal mol^{-1}, $\Delta H = -9$ kcal mol^{-1}, and $\Delta S = -18$ eu (Holladay, 1973). This negative value for ΔS agrees with that calculated from theoretical considerations as to loss of entropy upon protein dimerization (Steinberg & Scheraga, 1963).

The highly positive ΔS obtained for P-450 suggests that hydrophobic forces make the major contribution to self-association of the aggregate P_1. The observation that the molecular weight of P_1 increases with rising temperature between 10 and 26 °C also suggests that hydrophobic forces predominate in the association of monomers to form P_1. However, Gdn·HCl partially dissociates P_1, and nonionic detergents do not reduce P-450 to monomers; these observations suggest that other factors in addition to hydrophobic forces may contribute to P-450 aggregation.

The activity of P-450 does not appear to be a strict function of molecular weight, as evidenced by (1) lack of any effect on P-450 turnover number of protein concentration, which clearly determines the weight-average molecular weight (Figure 3, Table II), (2) the similar benzphetamine demethylase activities determined under various di-12 GPC and Triton N-101 concentrations (Figure 2), which also influence molecular weight, and (3) the similar sedimentation constants of the P-450 oxidase activity and protein concentration in the sedimentation velocity experiments. This situation is in clear contrast to that of mitochondrial D-β-hydroxybutyrate dehydrogenase, another membrane-associated enzyme activated by phospholipid monomers or vesicles, which is most active as a dimer and loses activity upon aggregation at higher concentrations (McIntyre et al., 1978). Both of these enzymes are somewhat unique in that they bind phospholipids in a noncooperative manner (Coon et al., 1976) and are activated by phospholipid monomers, in contrast to a number of other membrane-associated proteins such as cytochrome b_5 (Robinson & Tanford, 1975) and phospholipase A_2 (Bonsen et al., 1972).

The contribution of the small amount of residual nonionic detergent to these systems must be kept in mind; the possibility exists that this low level of detergent is responsible for maintaining the solubility of purified P-450. It should be noted that similar molecular weights have been estimated for rabbit P-450 preparations containing no nonionic detergent and less than 1 nmol of ionic detergent per nmol of P-450 and that electron microscopy of such preparations showed the absence of vesicular structures (Autor et al., 1973).

Finally, these results should be considered not only in reconstituted P-450 systems but also in postulating the spatial arrangement of the enzymes in the endoplasmic reticulum or in isolated microsomal membrane preparations. Any models that include the existence of P-450 monomers must also include energetic provisions for overcoming the attractive forces between P-450 monomers. Since P-450 monomers were not detected in the sedimentation equilibrium experiments without $NaDodSO_4$, the value of ΔG for the association of P-450 subunits to form P_1 cannot be easily determined. However, if an isodesmic association model is assumed and the lowest detectable P-450 concentration (50 nM, at which only an aggregate is detected) is considered, an association constant of $\geq 10^{13}$ M^{-1} and a resulting $\Delta G_{20°C}$ of ≤ 18 kcal mol^{-1} can be estimated for the association of P-450 monomers. While $NaDodSO_4$ is able to overcome these forces, nonionic detergents and di-12 GPC monomers and micelles are not, and the possibility must be considered that P-450 aggregates may exist within membranes under physiological conditions.

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[4] The following $\Delta G_{20°C}$ values have been calculated for association of other proteins: *Escherichia coli* glutamate decarboxylase, −4.2 kcal mol^{-1} (Teller, 1973); rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase, −4.2 kcal mol^{-1} (Teller, 1973); *Acinetobacter* glutaminase–asparaginase, −2.8 kcal mol^{-1} (Teller, 1973); bovine α-chymotrypsin, −3 kcal mol^{-1} (Teller, 1973); bovine insulin, −3 to −5 kcal mol^{-1} (Jeffrey & Coates, 1966); β-lactoglobulin A, −3.4 kcal mol^{-1} (Adams & Lewis, 1968); bovine procarboxypeptidase, −2.8 kcal mol^{-1} (Teller, 1973); corticotropin, +4 kcal mol^{-1} (Squire & Li, 1961); tobacco mosaic virus protein, −10.5 kcal mol^{-1} (Smith & Lauffer, 1967).

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