Characterization of the hydroxymethylglutaryl-CoA lyase precursor, a protein targeted to peroxisomes and mitochondria

Lyudmila I. ASHMARINA, Marie-France ROBERT, Marc-André ELSLIGER and Grant A. MITCHELL*

Service de génétique médicale, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec, Canada H3T 1C5

We previously showed that human liver hydroxymethylglutaryl-CoA (HMG-CoA) lyase (HL; EC 4.1.3.4) is found in both mitochondria and peroxisomes. HL contains a 27-residue N-terminal mitochondrial targeting sequence which is cleaved on mitochondrial entry, as well as a C-terminal Cys-Lys-Leu peroxisomal targeting motif. Because peroxisomal HL has a greater molecular mass and more basic pI value than mitochondrial HL, we predicted that peroxisomal HL retains the mitochondrial leader. To test this hypothesis, we expressed both the precursor (pHL) and mature (mHL) peptides in *Escherichia coli* and studied their properties. pHL purified by ion-exchange and hydrophobic chromatography had a pI of 7.6 on FPLC chromatofocusing and a molecular mass of 34.5 kDa on SDS/PAGE, similar to our findings for peroxisomal HL. For purified mHL, pI (6.2) and molecular mass (32 kDa) values resemble those of mitochondrial HL. Purified pHL is similar to mHL in K_m for HMG-CoA (44.8 μM), k_{cat} (6.3 min^{-1}) and pH optimum (9.0–9.5). However, the quaternary structures of pHL and mHL differ. On Superose 12 FPLC gel filtration and also on ultrafiltration, both in the presence and in the absence of HMG-CoA, pHL behaves as a monomer whereas mHL migrates as a dimer. We conclude that the HL precursor is probably identical to peroxisomal HL, that its catalytic properties resemble those of mature mitochondrial HL, and that the mitochondrial leader peptide prevents dimerization of pHL.

INTRODUCTION

3-Hydroxy-3-methylglutaryl-CoA lyase (HL; EC 4.1.3.4) catalyses the cleavage of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to acetoacetic acid and acetyl-CoA in the mitochondrial matrix, the last step of both ketogenesis and of leucine catabolism. Mitochondrial HL is a 68.8 kDa homodimer expressed in all tissues studied to date. In humans the autosomal recessively inherited deficiency of HL leads to episodes of coma and hypoglycaemia which can resemble Reye syndrome and sudden infant death syndrome [1]. We cloned human, mouse and chicken liver HL cDNAs and have isolated mouse and human HL genomic clones [2,3]. Both the human and mouse HL precursors contain 325 residues including a 27-residue N-terminal mitochondrial leader and the C-terminal tripeptide Cys-Lys-Leu, a potential peroxisomal targeting motif. We recently showed the presence of active HL in peroxisomes isolated from mouse and human livers [4]. Peroxisomal HL has a molecular mass ~2.5 kDa greater than mitochondrial HL and also has a more basic pI, which led us to hypothesize that structurally, peroxisomal HL is identical to the HL precursor peptide, in which the mitochondrial leader is retained. To test this hypothesis we expressed, purified and characterized both recombinant HL precursor (pHL) and mature HL (mHL) and compared their properties with those of peroxisomal and mitochondrial HLs.

MATERIALS AND METHODS

Construction of recombinant expression plasmids

The pTrcHL-C323S plasmid [5], kindly provided by H. Miziorko, was used for expression of mHL and for construction of the pHL expression vector. It contains the full coding sequence of the mature human HL cDNA, preceded by Met and Gly codons corresponding to the *Nco*I cloning site. In this plasmid the codon of the reactive Cys-323 residue has been mutated to a serine codon. The peptide expressed from pTrcHL-C323S is more stable than wild-type HL, but its catalytic properties have been shown to be close to those of purified recombinant wild-type human HL and of purified chicken liver HL [5].

To modify the pTrcHL plasmid for expression of pHL we first amplified a cassette containing the 5′ extremity of the pHL cDNA, using as template pHLH-1, a plasmid containing the full-length human HL cDNA [2]. The 5′ and 3′ oligonucleotide primers were respectively 5′-AACCATGGCAGCAATG-AGGAA-3′, which contains the initiation methionine codon of HL within the *Nco*I cloning site (underlined), and 5′-TTGATGTT-CTTCTTGGT-3′. The reaction mixture contained 1 ng of pHLH-1, 50 μM (final concentration) of each dNTP, 0.4 μg of each primer, 2.5 units of *Taq* DNA polymerase (Bethesda Research Laboratories), 20 mM Tris/HCl, pH 8.3, 100 mM KCl and 2.5 mM $MgCl_2$ in a total volume of 50 μl. Amplification cycles were: 15 s at 94 °C, 15 s at 56 °C and 30 s at 72 °C for 30 cycles, followed by a 6-min extension at 72 °C. The 422-residue amplified fragment, which contains an internal *Sst*I site, was digested with *Nco*I and *Sst*I and cloned into the pTrcHL-C323S vector, from which the corresponding cassette had been excised. The resulting plasmid was sequenced on both strands over the entire *Nco*I–*Sst*I cassette. With the exception of the C323S substitution, the recombinant pHL peptide expressed from pTrc-pHL is predicted to be identical to wild-type pHL, which fortuitously also contains a Met-Gly dipeptide at its N-terminus.

Expression and purification of recombinant human HL

The resulting expression plasmid was transformed into *Escherichia coli* strain K12PR745 (New England Biolabs). Bacteria were grown at 37 °C in nutrient broth [containing Trypticase

Abbreviations used: DTT, dithiothreitol; HL, hydroxymethylglutaryl-CoA lyase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; mHL, mature HL; pHL, HL precursor.

* To whom correspondence should be addressed.

Peptone, 10 g/l; BBL Yeast Extract, 5 g/l (both from Becton Dickinson) and NaCl, 5 g/l] supplemented with ampicillin (50 mg/l). After the cultures reached an absorbance of 0.8–1.4 at 600 nm, expression of HL was induced by addition of isopropyl-1-thio-β-δ-galactoside to a final concentration of 1 mM. Cells were harvested after overnight growth at 28 °C and centrifuged at 7000 ***g*** for 10 min at 4 °C.

We purified mHL or pHL from the pellet of a 1-litre culture as described by Roberts et al. [5] with the following three modifications. First, cells were disrupted by sonication (3 times for 60 s at 60 W, using a Sonic dismembrator, Fisher-Scientific), rather than by use of a French press. Secondly, the eluate of the Q-Sepharose column was directly adjusted to 1 M $(NH_4)_2SO_4$, cleared by centrifugation at 10000 ***g*** for 30 min and then applied to the Phenyl-Sepharose column, without performing $(NH_4)_2SO_4$ fractionation. Thirdly, gel filtration was performed on an FPLC Superose 12 HR column (Pharmacia Biotech) instead of a Superose 12 column.

Enzyme and protein assays and determination of kinetic constants

HL was assayed spectrophotometrically using *R*,*S*-HMG-CoA (Sigma) as a substrate [6]. To determine the pH-dependence of HL activity, the pH of the reaction mixture was varied between 7.0 and 10.0 using a buffer containing 50 mM glycyl-glycine, 50 mM glycine, 20 mM $MgCl_2$ and 20 mM dithiothreitol (DTT). One unit of enzyme activity is defined as the conversion of 1 μmol of substrate per min. Protein determination was performed according to the method of Bradford [7] with BSA (Sigma) as a standard.

K_m and k_{cat} values were computed from the dependency of the initial reaction rate on the substrate concentration by non-linear regression, using the Michaelis–Menten equation. The kinetic constants were calculated using half of the total concentration of *R*,*S*-HMG-CoA, since only the *S*-form is biologically active.

SDS/PAGE

Electrophoresis was performed on an SDS/polyacrylamide gel (11 %, w/v) under reducing conditions according to the method of Laemmli [8]. Protein staining was performed with Coomassie Blue R-250 (Sigma) followed, if necessary, by silver staining using the Fast Silver Staining Kit (Bio-Rad).

Immunoblotting

Western blotting was performed as described using a rabbit anti-recombinant human HL serum [4].

Chromatofocusing

Purified HL preparations were dialysed against 25 mM BisTris buffer (Sigma), pH 7.6. Following the manufacturer's protocol, 1.0 ml of dialysate was applied to an FPLC Mono P column (Pharmacia Biotech), equilibrated with the above buffer, and eluted with 50 ml of Polybuffer 74 (Pharmacia Biotech). Fractions were collected and assayed for HL activity.

Gel filtration

Purified preparations of HL were dialysed against 50 mM sodium phosphate, 0.1 M NaCl, 10 % (v/v) glycerol and 1 mM DTT, pH 7.8. The preparation was concentrated to 200 μl in a concentrating cell (Amicon) equipped with a PM-10 membrane. The samples were then applied to an FPLC Superose 12 HR column (Pharmacia Biotech) equilibrated with the above buffer. The flow rate was 0.3 ml/min. Fractions (0.5 ml) were collected, assayed for HL activity and examined by SDS/PAGE. The molecular masses of the eluted peptides were calculated using a calibration curve obtained with the following protein standards (all from Pharmacia Biotech): ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (69 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (12.6 kDa).

Ultrafiltration

Ultrafiltration of recombinant pHL and mHL was performed in a concentrating cell (Amicon) equipped with a XM-50 membrane at 4 °C and an operating pressure of 3.2 bar. The purified recombinant HL preparation (0.1 mg of protein in 1 ml of buffer, containing 25 mM glycyl-glycine, 25 mM glycine, 10 mM $MgCl_2$ and 2 mM DTT, pH 9.25, with or without 2 mM HMG-CoA) was applied to the cell and concentrated to 0.1 ml. HL activity was assayed in the flow-through and in the concentrate, and the fraction of HL activity retained by the membrane was calculated. The retentions of pHL and mHL were compared with those which we found for the following proteins (all from Pharmacia Biotech): BSA, 69 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa; ribonuclease, 12.6 kDa.

RESULTS AND DISCUSSION

Expression of recombinant pHL and mHL

Crude lysates of cells transformed with pTrc-pHL contained immunoreactive and enzymically active HL. On immunoblots (Figure 1), we observed a signal at ~34.5 kDa, the intensity of which was approximately equal to that of the 32 kDa mHL peptide from *E. coli* transfected with pTrcHL-C323S. Similar amounts of HL activity were present in crude lysates of cells transformed with either pTrc-pHL or pTrcHL-C323S.

Using a method similar to that of Roberts et al. [5] we successfully purified enzymically active, electrophoretically homo-

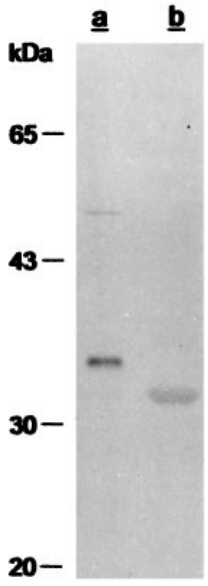

Figure 1 Immunoblot of crude bacterial expression lysates for (a) pHL and (b) mHL

Samples (10 μl) of lysate were subjected to SDS/PAGE, transferred to an Immobilon membrane and incubated with polyclonal rabbit anti-(human HL) antibodies.

Table 1 Purification of recombinant pHL and mHL

	pHL				mHL			
	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Soluble extract	133	2.8	1	100	140	4.6	1	100
Q-Sepharose	18.1	19	6.8	92	22.5	24	5.2	84
Phenyl-Sepharose	0.42	76.9	27.5	8.6	1.4	84	18.2	12
Superose 12 HR	0.1	220	78.6	6.1	0.144	249	54.1	5.6

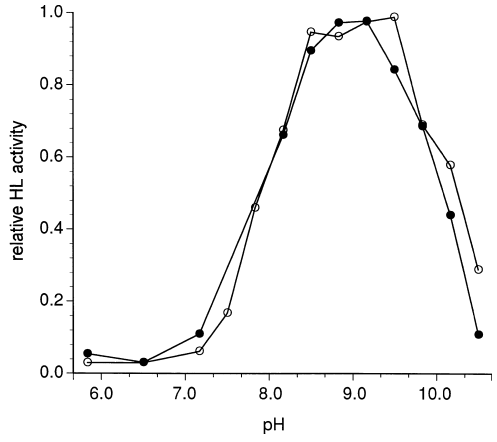

Figure 2 pH dependence of pHL and mHL activity

Samples (5 μg) of purified recombinant pHL (○) or mHL (●) were used in each assay. The point with the highest HL activity was arbitrarily assigned a value of 1.0.

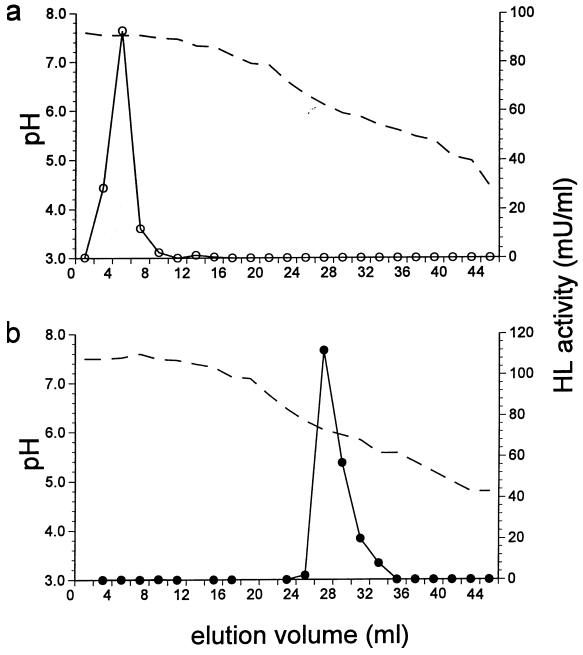

Figure 3 FPLC chromatofocusing of (a) pHL and (b) mHL using a Mono P column

The HL activity (solid lines) and pH profile (dashed line) of the eluate are shown.

geneous recombinant pHL and mHL. This confirms the results of Roberts et al. with respect to mHL purification and shows that the same method can be extended to purification of pHL (Table 1). We detected two differences in the behaviour of pHL and mHL during purification. First, the elution volume from the Q-Sepharose column was 30 ml for pHL in contrast to 37 ml for mHL. Secondly, on gel filtration, pHL eluted later than mHL as discussed below.

Enzymic properties of recombinant pHL

The specific activities of pHL and mHL are similar to each other (Table 1) and to published data for recombinant human HL and purified avian HL [5,9]. The K_m for HMG-CoA was 44.8 ± 4 μM for pHL (mean $\pm$ S.D., $n = 3$) and 44.5 ± 0.7 μM for mHL, the later agreeing with published values [5]. k_{cat} values were 6.3 ± 0.1 min^{-1} for pHL and 7.6 ± 0.1 min^{-1} for mHL. We conclude that recombinant human pHL and mHL have similar affinity and catalytic activity toward HMG-CoA. The K_m value is of the same order as that reported for recombinant human HL containing the wild-type Cys-323 residue (25 μM [5]), and for HL purified from bovine, pig and chicken livers (3.5 to 25 μM [9–11]).

We observed an alkaline optimal pH (9.0–9.5) for both pHL and mHL (Figure 2), as has been reported for HL from human fibroblasts [6], pig heart [12], bovine [10] and chicken liver [9], *Tetrahymena pyriformis* [13] and *Pseudomonas mevalonii* [14]. Our estimate that 6 % of liver HL activity is peroxisomal [4], was based upon *in vitro* assays at pH 9.0. This value probably overestimates the *in vivo* capacity for HMG-CoA lysis in peroxisomes, because HL activity is predicted to be enhanced by the high pH of the mitochondrial matrix ($\sim$8) [15]. However, this small catalytic capacity of peroxisomal HL may not reflect its biological importance, since its function, if any, may differ from that of mitochondrial HL.

Chromatofocusing of recombinant pHL and mHL

On FPLC chromatofocusing analysis (Figure 3) we observed a difference in pI between pHL ($\geqslant$7.6) and mHL (6.1), which may explain their different behaviour on ion-exchange chromatography, described above. We previously estimated the pI values of mouse liver peroxisomal and mitochondrial HL as $\geqslant$7.3 and 6.4 respectively [4]. Therefore, both the pI and molecular-mass values of recombinant human pHL are similar to those measured for peroxisomal HL, supporting the hypothesis that peroxisomal HL is identical to the HL precursor.

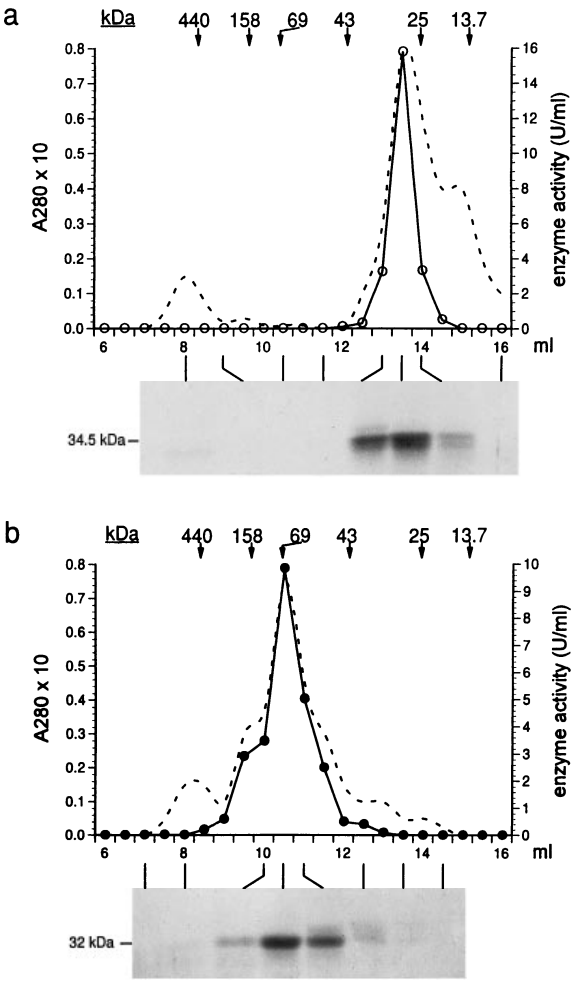

Figure 4 Gel filtration and SDS/PAGE analysis of (a) pHL and (b) mHL

The upper panels of (**a**) and (**b**) show the FPLC Superose 12 HR gel-filtration profile of HL activity (solid line) and total protein (dashed lines). Samples of pHL (100 μg) (**a**) and of mHL (140 μg) (**b**) were analysed, as described in the Materials and methods section. The positions of the elution peaks of the molecular mass standards described in the Materials and methods section are shown. The lower panels show silver-stained SDS/PAGE analysis of the indicated gel-filtration fractions. 100 μl of each fraction were used.

Gel- and ultrafiltration of recombinant pHL and mHL

As mentioned above, we found a marked difference between the properties of pHL and mHL at the gel-filtration step of purification. mHL elutes with an apparent molecular mass of 65.0 ± 5.0 kDa (Figure 4), suggesting that it is a homodimer, in agreement with previous studies of HL purified from chicken liver [9], purified recombinant human mHL [5], and early studies with partially purified pig [11] and bovine liver [10] HL. In contrast, the apparent molecular mass of pHL (Figure 4a) is 34.1 ± 2.0 kDa, close to that of monomeric pHL (34.4 kDa). We also performed ultrafiltration of purified pHL and mHL (Table 2), using an XM-50 membrane which retains proteins of molecular mass greater than about 50 kDa. As expected, mHL was completely retained, whereas 57% of pHL traversed the membrane, suggesting a molecular mass between 25 and 43 kDa (Table 2). We also performed ultrafiltration at pH 9.25 in the presence of 2 mM HMG-CoA, the conditions used in our HL assay, and found no change in the percentage either of pHL or of mHL retained. Therefore, pHL behaves as a monomer, and mHL as a dimer, both at pH 7.8 and 9.0, and in the presence or absence of substrate.

Table 2 Retention of HL and protein standards following ultrafiltration on an XM-50 membrane

Protein	Retained fraction (%)*
HL peptides	
mHL	100 ± 0
mHL + HMG-CoA	100 ± 0
pHL	43 ± 5
pHL + HMG-CoA	42 ± 6
Standards	
BSA (69 kDa)	96 ± 3
Ovalbumin (43 kDa)	70 ± 5
Chymotrypsinogen (25 kDa)	31 ± 4
Ribonuclease (12.6 kDa)	14 ± 4

* The means $\pm$ S.D. of three determinations are shown.

We conclude that pHL is an enzymically active monomer and that the presence of the mitochondrial leader peptide does not markedly influence the catalytic properties of human HL. Studies to date of vertebrate HL are consistent with it being a dimer [5,9]. Furthermore, although bacterial HL was initially reported to be a monomer [13,14] purified recombinant bacterial HL behaves as a dimer [16]. pHL is therefore the only known HL which does not dimerize under any conditions tested. In previous studies with HL purified from natural sources, monomeric HL was not detected, probably because pHL accounts for only $\sim 6\%$ of tissue HL [4]. All data are consistent with the hypothesis that peroxisomal HL is a catalytically active monomer structurally identical to the mitochondrial HL precursor. Formal proof of this will require purification of peroxisomal HL and N-terminal peptide sequencing. Our studies also show that dimerization is not essential for HL catalytic activity.

Although our observation may be applicable only to HL, we speculate that the leader sequence of other mitochondrial enzymes may also directly affect quaternary structure, possibly facilitating mitochondrial import or modulating enzyme regulation. As a preliminary test of the potential effect of mitochondrial presequences upon quaternary structure, we searched the Protein Data Base [17] for the structures of mitochondrial matrix peptides which crystallize as oligomers. We found 10 such enzymes. (The 10 enzymes and their Protein Data Base identification codes are: aspartate aminotransferase, 9aat; 1bcd; citrate synthase, 5csc; dihydrolipoamide dehydrogenase, 3lad; glutamine synthase, 2gls; isocitrate dehydrogenase, 9icd; malate dehydrogenase, 1mld; medium-chain fatty acyl-CoA dehydrogenase, 3mdd; nucleoside kinase, 2nck; succinyl-CoA synthetase, ADP-forming, 1scu; and manganese superoxide dismutase, 3mds.) In four out of 10 structures, the N-terminus either directly contacts another subunit (aspartate aminotransferase, medium-chain fatty acyl-CoA-dehydrogenase) or is part of a subdomain which is in contact with another subunit (malate dehydrogenase, dihydrolipoamide dehydrogenase). The effect of mitochondrial leader sequences on subunit interactions in these and other proteins can be tested directly using recombinant precursor peptides.

We thank Henry Miziorko for ongoing collaboration and for supplying the pTrc HL-C3235 vector, Alexey V. Pshezhetsky for helpful advice and Micheline Patenaude for

excellent secretarial assistance. This work was funded by a grant from the Medical Research Council of Canada to G.A.M. L.I.A. received a visiting professor bursary from the Fonds de la Recherche en Santé du Québec.

REFERENCES

1 Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. (1994) The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York
2 Mitchell, G. A., Robert, M.-F., Hruz, P. W., Fontaine, G., Behnke, C. E., Mende-Mueller, L. M., Wang, S., Shaeppert, K., Lee, C., Gibson, K. M. and Miziorko, H. (1993) J. Biol. Chem. **268**, 4376–4381
3 Wang, S., Nadeau, J. H., Duncan, A., Robert, M.-F., Fontaine, G., Schappert, K., Johnson, K. R., Zietkiewicz, E., Hruz, P., Miziorko, H. and Mitchell, G. A. (1993) Mammalian Genome **4**, 382–387
4 Ashmarina, L. I., Rusnak, N., Miziorko, H. M. and Mitchell, G. A. (1994) J. Biol. Chem. **269**, 31929–31932
5 Roberts, J. R., Narasimhan, C., Hruz, P. W., Mitchell, G. A. and Miziorko, H. M. (1994) J. Biol. Chem. **269**, 17841–17846
6 Wanders, R. J. A., Schutgens, R. B. H. and Zoeters, P. H. M. (1988) Clin. Chim. Acta **171**, 95–102
7 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
8 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
9 Kramer, P. R. and Miziorko, H. M. (1980) J. Biol. Chem. **25**, 11023–11028
10 Stegink, L. D. and Coon, M. J. (1968) J. Biol. Chem. **243**, 5272–5279
11 Higgins, J. P., Kornblatt, J. A. and Rudney, H. (1972) The Enzymes, Academic Press, New York
12 Bachhawat, B. K., Robinson, W. G. and Coon, M. J. (1955) J. Biol. Chem. **216**, 727–736
13 Premkala, P. and Holmlund, C. E. (1987) Int. J. Biochem. **19**, 385–389
14 Scher, D. S. and Rodwell, V. W. (1989) Biochim. Biophys. Acta **1003**, 321–326
15 Tzagoloff, A. (1982) Mitochondria, Plenum Press, New York
16 Narasimhan, C. and Miziorko, H. M. (1992) Biochemistry **31**, 11224–11230
17 Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. **112**, 535–542

Received 4 September 1995/10 November 1995; accepted 21 November 1995