Δ^3, Δ^2 -Enoyl-CoA isomerase is the protein that copurifies with human glutathione S-transferases from S-hexylglutathione affinity matrices

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An unidentified 30 kDa protein frequently copurifies with human glutathione S-transferases from S-hexyl-glutathione affinity matrices. Application of two-step sequential affinity chromatographic methods yielded a homogeneous preparation of that protein from human liver specimens. The protein was digested with *Achromobacter* protease I, and sequences of peptides

resolved by h.p.l.c. showed a high degree of identity with those of rat mitochondrial Δ^3, Δ^2 -enoyl-CoA isomerase. The human protein also exhibited catalytic activity with Δ^3 -cis-octenyl CoA as a substrate. Thus it is identified as liver Δ^3, Δ^2 -enoyl-CoA isomerase.

INTRODUCTION

Human glutathione S-transferase (GST) preparations frequently contain additional proteins that copurify with the GSTs from S-hexyl-glutathione affinity matrices. These components, which have somewhat larger molecular masses than any of the GST subunits, have been found in tissue extracts and cultured cells. Tu et al. (1986) observed polypeptide bands with molecular masses greater than 30 kDa during SDS/PAGE analysis of human liver GSTs. By a series of chromatographic steps used to resolve GST subunits, Hussey et al. (1991) achieved a partial purification of a higher-molecular-mass component from human skeletal muscle extracts which had no catalytic activity with 1-chloro-2,4-dinitrobenzene, a general GST substrate (Listowsky, 1993). No further attempts were made to characterize the protein. Castro et al. (1990) reported that antibodies against class- α GSTs cross-reacted weakly with a 30 kDa protein eluted from S-hexyl-GSH affinity columns. Of greater significance, those authors found striking variations in levels of the protein in different human cell lines (Castro et al., 1990). In spite of its occurrence in numerous GST preparations, the protein has not been identified. It has been known for some time that glyoxylase often coelutes with GSTs from GSH affinity matrices (Hayes, 1988; Hao et al., 1994), but this is not surprising because glyoxylases utilize GSH for catalytic function. It is not clear, however, whether the 30 kDa protein is also a GSH-linked protein, which would account for its adherence to the S-hexyl-GSH affinity columns.

In this report, a simple two-step procedure is described for purification of the 30 kDa protein that binds to S-hexyl-GSH affinity matrices. The protein is identified as Δ^3, Δ^2 -enoyl-CoA isomerase (EC 5.3.3.8) on the basis of sequence analysis of peptide digests. The implications of these results are relevant to studies on this key enzyme of fatty acid β -oxidation, because major changes in this component have been observed in different cell types and in malignant tissue (Kelley et al., 1994).

MATERIALS AND METHODS

Protein purification

Human liver specimens were obtained 10–12 h *post mortem* from 28- and 46-year-old male subjects with no evidence of pre-

existing disease who died in motor vehicle accidents. Each tissue specimen was processed separately. Frozen tissue (20 g) was homogenized in buffer A (10 mM Tris/HCl buffer, pH 7.9, containing 0.1 M NaCl and 0.1 mM dithiothreitol). After centrifugation at 20 000 g, the supernatant fraction was applied directly to an S-hexyl-GSH agarose affinity column (5 cm $\times$ 1.0 cm) (Sigma). The column was washed with buffer A, and the bound fraction was eluted with 5 mM S-hexyl-GSH (Sigma) in 50 mM Tris/HCl, pH 9.6. The eluate was dialysed against buffer A and subjected to Sephadex-G25 gel-permeation chromatography to remove excess S-hexyl-GSH. The protein was concentrated by ultrafiltration and applied to an epoxy-activated agarose linked to GSH affinity column (Sigma) (Simons and VanderJagt, 1977). The flow-through fraction of the second affinity matrix, which contained a 30 kDa polypeptide, was collected, and the GSTs were then eluted from the column with 10 mM GSH (Sigma) in 50 mM Tris/HCl, pH 9.6.

Enzyme assays and protein characterization

GST enzymatic activities were determined with 1 mM 1-chloro-2,4-dinitrobenzene (Aldrich) and 1 mM GSH as substrates, by measuring A_{343} in 10 mM sodium phosphate, pH 6.7 (Jensson et al., 1985). Δ^3, Δ^2 -Enoyl-CoA isomerase activity was measured based on the coupled assay system described by Stoffel et al. (1964) and Binstock and Schulz (1981). The assay mixture (1.0 ml) contained 0.325 mM NAD⁺, 0.1 mM CoASH, 52 μ M Δ^3 -cis-octenyl CoA, 0.2 mg/ml BSA and 0.08 % (v/v) Triton X-100 in 0.2 M potassium phosphate, pH 8.0. The Δ^3, Δ^2 -enoyl-CoA isomerase reaction was coupled to beef liver crotonase (4 i.u./ml), pig heart 3-hydroxyacyl-CoA dehydrogenase (0.3 i.u./ml) and 3-ketoacyl-CoA thiolase (0.04 i.u./ml) reactions, and the reaction was followed by the appearance of NADH (A_{340}) produced in the 3-hydroxyacyl-CoA dehydrogenase reaction.

PAGE was performed using 12 % (w/v) acrylamide slab gels, which were then stained with Coomassie Blue. Purity of the 30 kDa protein was determined by reversed-phase h.p.l.c. using a Hewlett–Packard 1090 liquid chromatograph and an Applied Biosystems C₄ column (2.1 mm $\times$ 100 mm).

Abbreviation used: GST, glutathione S-transferase.

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Proteolytic cleavage and analysis of peptides

The protein was digested with *Achromobacter* protease I for 12 h at 37 °C in 50 mM Tris/HCl, pH 8.8, containing 3 M urea. The ratio of protease, which selectively cleaves C-lysyl bonds, and protein substrate was approx. 1:200 (w/w). The peptides generated were resolved and purified by reversed-phase h.p.l.c. using a Spectra-Physics model SP9900/8780/8500/8773/4290 chromatograph equipped with a Barspec model 1937 chrom-A-scope fast scan detector. A linear gradient of 5–65% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid was run for 80 min., using an Aquapore-300 (C₈) column (4.6 mm × 220 mm). Sequence analyses of the isolated peptides were determined using a Beckman model LF3400 Sequencer.

Sequence data comparisons

The Swiss-Prot and EMBL databases of GCG (Genetics Computer Group, Program Manual for GCG package, version 7, 575 Science Drive, Madison, WI, April 1991) were surveyed to detect amino acid sequence identities using the T FASTA program. Sequences were aligned using GAP and PILEUP programs.

RESULTS

Purification and characterization

Cytosolic extracts from human liver were applied to *S*-hexyl-GSH agarose affinity matrices and the columns were eluted with 5 mM *S*-hexyl-GSH. SDS/PAGE patterns of the eluant revealed components ordinarily ascribed to human GST subunits (Takahashi et al., 1993), as well as a band corresponding to a molecular mass of 30 kDa (Figure 1a). After removal of excess *S*-hexyl-GSH eluant, the protein fraction bound by the *S*-hexyl-GSH matrix was then applied to a GSH-agarose affinity column. The 30 kDa component was not retained by the second affinity column, and was thereby separated from the bound GSTs. The GSTs were subsequently eluted with GSH (Figure 1a). Thus, sequential utilization of the two GSH affinity matrices yielded a highly purified 30 kDa polypeptide (Figure 1b). Reversal of the procedure by application of the flow-through fraction from the GSH affinity matrix to a *S*-hexyl-GSH matrix yielded the same 30 kDa component that was eluted with 5 mM *S*-hexyl-GSH. The protein preparations were primarily single peaks on h.p.l.c., although a shoulder was resolved in some preparations (Figure 1b).

The higher-molecular-mass protein cross-reacted weakly with anti-(α -GST) antisera in immunoblot analysis (results not shown). However, the fractions containing the 30 kDa subunits were devoid of catalytic activity with 1-chloro-2,4-dinitrobenzene, a common substrate for most GSTs (Mannervik and Danielson, 1988). Isoelectric focusing yielded a single component with a *pI* of 5.8. Because this component corresponds in all these respects to the undefined entity found in previous studies on human GSTs, and the sequential application of the affinity matrices afforded a highly purified protein, efforts were made to identify this protein.

Identification of the 30 kDa protein as Δ^3,Δ^2 -enoyl-CoA isomerase

Edman degradation generated a sequence (FGXQRLVEP) in low yield, but the main portion of the 30 kDa protein was refractory to the N-terminal sequence analysis. The protein was therefore digested with *Achromobacter* protease I which preferentially cleaves C-terminal to lysyl residues. The peptide fragments were resolved by h.p.l.c. (Figure 2). Seven well defined peptides for which unambiguous sequences could be determined

Figure 1 (a) Purification by GSH-affinity chromatography and (b) reversed-phase h.p.l.c. analysis of a 30 kDa protein

(a) Human liver extracts (see the Materials and methods section) were applied to *S*-hexyl-GSH affinity matrices that were eluted with 5 mM *S*-hexyl-GSH (lane 1). The eluate was then passed through an epoxy-GSH affinity matrix and the bound fraction (lane 2) was eluted with 10 mM GSH. Lane 3 shows the protein fraction bound by the *S*-hexyl-GSH affinity matrix but not bound (flow-through fraction) by sequential application to the epoxy-GSH matrix. Lane M contains the molecular-size markers (Bio-Rad). Lanes 1–3 were deliberately overloaded with protein [50 µg/lane (lanes 1 and 2) and 25 µg/lane (lane 3)], as indications of protein purity. Lanes 4 and 5 are the same as 2 and 3 respectively, but contained only 10 µg of protein/lane. (b) The 30 kDa fraction obtained by sequential affinity chromatography (see above) was resolved by reversed-phase h.p.l.c. on an Applied Biosystems C₄ column (2.1 mm × 100 mm) using a Hewlett Packard 1090 Liquid Chromatograph. A linear gradient of 0–100% (v/v) acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid was run over a period of 40 min.

were selected for sequence analysis. None of the sequences showed identity to any of the GSTs characterized so far.

The sequence of peptide 2 (see Figure 2) corresponded to that of the N-terminal sequence of the intact protein. The peptide sequences were compared with sequences in the Swiss-Prot and EMBL protein databases. All 23 residues of peptide 6 were identical to a corresponding segment of rat mitochondrial Δ^3,Δ^2 -enoyl-CoA isomerase. This peptide had a lysine at its penultimate N-terminal position, possibly because of incomplete digestion by the *Achromobacter* protease I. Peptide 2 also showed high sequence identity with the Δ^3,Δ^2 -enoyl-CoA isomerase (12 of 20 residue identity), and would correspond to the N-terminal sequence of the protein which had been processed with its mitochondrial targeting leader sequence cleaved (Müller-Newen and Stoffel, 1991; Palosaari et al., 1991; Kilponen et al., 1992; Tomioka et al., 1992). The 28 residues of peptide 5 only had 4 substitutions, whereas 11 of 14 residues of peptide 1 and 8 of 11 residues of peptide 4 were identical. Peptide 3 had a stretch of 5 divergent amino acids with only 7 of 15 identical to the rat Δ^3,Δ^2 -enoyl-CoA isomerase. The sequence of the 30 kDa protein also differed from that of a GST isolated from rat mitochondrial matrix (Harris et al., 1991).

Figure 3 shows a likely sequence alignment of the peptides obtained by digestion of the 30 kDa component and the sequence deduced from the cDNA for rat mitochondrial Δ^3,Δ^2 -enoyl-CoA isomerase. Although sequences for the corresponding human enoyl-CoA isomerase are currently not available, the extent of

Figure 2 Resolution of peptides obtained by cleavage of the 30 kDa protein with *Achromobacter* protease I by reversed-phase h.p.l.c.

Conditions for proteolysis and h.p.l.c. are described in the Materials and methods section. The peptides selected for sequence analysis are numbered 1–7.

sequence alignment and apparent size of the protein provide strong evidence to support the conclusion that the 30 kDa protein bound to *S*-hexyl-GSH affinity matrices is human liver Δ^3, Δ^2 -enoyl-CoA isomerase.

To verify the identity of the protein and determine whether the enzyme isolated in this manner retains its catalytic function,

Δ^3, Δ^2 -enoyl-CoA isomerase assays were performed. Indeed, the human enzyme purified using GSH affinity methods exhibited a specific activity of 12 units/mg with Δ^3 -*cis*-octenyl CoA as a substrate (Stoffel et al., 1964; Binstock and Schulz, 1981). Because the enzyme specifically adhered to *S*-hexyl-GSH affinity matrices, it seemed likely that *S*-hexyl-GSH is bound by the enzyme. This ligand was therefore tested for its effect on the catalytic activity of the enzyme; however, even at concentrations as high as 1 mM, *S*-hexyl-GSH had no inhibitory effect.

DISCUSSION

The 30 kDa protein that consistently copurifies with human GSTs has confounded studies on these proteins ever since *S*-hexyl-GSH affinity matrices came into routine use for the isolation of GSTs (Mannervik and Guthenberg, 1981). In particular, its occurrence in variable amounts from different sources, and its cross-reactivity with anti-(α -GST) antisera has attracted considerable attention (Tu et al., 1986; Castro et al., 1990; Howie et al., 1990; Hussey et al., 1991). Conclusive evidence is presented here to identify this protein as Δ^3, Δ^2 -enoyl-CoA isomerase. The convenient two-step sequential affinity chromatographic methods yield a highly purified preparation of the protein; they are also less cumbersome than procedures currently used to isolate Δ^3, Δ^2 -enoyl-CoA isomerase from mitochondria (Binstock and Schultz, 1981).

Although sequence data for human Δ^3, Δ^2 -enoyl-CoA isomerase are currently not available, sequences of the peptide fragments (Figure 2) which encompass 142 amino acid residues of the protein, share 78% sequence identity with the deduced sequence of rat mitochondrial Δ^3, Δ^2 -enoyl-CoA isomerase (Müller-Newen and Stoffel, 1991; Tomioka et al., 1992; Babbitt et al., 1992; Kamijo et al., 1993; Müller-Newen and Stoffel, 1993). This explains why antibodies against the rat enzyme cross-react with the human isomerase (Palosaari et al., 1990; Kilponen and Hiltunen, 1993). Moreover, the size of the protein subunit, 30 kDa in SDS/polyacrylamide gels (Figure 1), is consistent with

Figure 3 Sequence alignment of peptides of the human liver 30 kDa protein and rat mitochondrial Δ^3, Δ^2 -enoyl-CoA isomerase

The sequence of the rat enzyme (R-ECI) (Palosaari et al., 1991; Tomioka et al., 1992) and those obtained for the human liver protein (H-ECI) are aligned. The amino acid identities are indicated by white letters on a black background. Peptides are numbered according to the h.p.l.c. pattern of Figure 2. Residues of the putative mitochondrial targeting leader sequence are indicated by italics.

that of the rat counterpart. The catalytic activity of the enzyme with Δ^3 -*cis*-octenyl CoA reaffirms, with a high degree of certainty, that the protein is indeed human liver Δ^3, Δ^2 -enoyl-CoA isomerase.

It is interesting to note that some GST isoforms have Δ^5 -3-ketosteroid isomerase activities (Benson et al., 1977). The conversion of Δ^5 -3-ketosteroids to the conjugated Δ^4 -3-ketosteroids is a reaction similar to that associated with the Δ^3, Δ^2 -enoyl-CoA isomerases. In addition, a number of studies have indicated that the 30 kDa band cross-reacts with anti-GST antisera on immunoblots (Tu et al., 1986; Castro et al., 1990). On the basis of primary structure comparisons of the sequences available thus far (Buetler and Eaton 1992; Daniel, 1993), it is not clear why antibodies raised against GSTs should cross-react with Δ^3, Δ^2 -enoyl-CoA isomerase. It seems reasonable to conclude that contamination of GST antigen preparations with enoyl-CoA isomerase accounts for its weak cross-reactivity. However, Tu et al. (1986) found that antisera raised against rat liver and testis and sheep GSTs, which did not contain the higher molecular weight bands, also precipitated one of the human 30 kDa subunits. Thus, the basis for the immunological reaction is still uncertain.

The enzyme adheres to *S*-hexyl-GSH affinity matrices and is specifically eluted with *S*-hexyl-GSH, suggesting that this ligand is bound to the protein. Because *S*-hexyl-GSH does not inhibit the catalytic activity of the Δ^3, Δ^2 -enoyl-CoA isomerase, it is possible that binding occurs at a domain other than the catalytic site. Alternatively, the types of interactions between immobilized *S*-hexyl-GSH with Δ^3, Δ^2 -enoyl-CoA isomerase may not reflect those that occur in solution. Even with GSTs themselves, the behaviour of immobilized GSH analogues sometimes differs from that predicted on the basis of solution properties (Lyttle et al., 1994). Binding specificity may also be determined, in part, by the alkyl side chain of the *S*-hexyl GSH, and may involve lipophilic regions of the protein that span the mitochondrial membrane. Emerging structural studies on the isomerases (Zeelen et al., 1992) should shed light on the nature of the interactions.

Because of the weak cross-reactivity of the 30 kDa protein with anti-GST antisera, Castro et al. (1990) tentatively considered the possibility that it is an additional class- α GST not previously described. The protein is particularly abundant in HepG2 hepatoma and K562 erythroleukemia cells, and was also found in Raji Burkitt lymphoma, LS174T colon carcinoma and Chang cells, but was not detected in some other cell lines (Lewis et al., 1989; Castro et al., 1990; Howie et al., 1990). It may now be deduced that those results reflect levels of Δ^3, Δ^2 -enoyl-CoA isomerase in the cell lines. In addition, a protein has been resolved by hexyl GSH affinity chromatography of extracts from normal-breast and breast-cancer tissue specimens (Kelley et al., 1994). The human liver Δ^3, Δ^2 -enoyl-CoA isomerase described in this report was found to coelute upon reversed-phase h.p.l.c. with the corresponding fraction from the breast tissues (L. M. Kauvar, personal communication). Although detected in all breast samples, this protein exhibited variable levels in different subjects, and in many cases, the tumour specimens had elevated levels of Δ^3, Δ^2 -enoyl-CoA isomerase relative to matched normal breast tissue. The results suggest that Δ^3, Δ^2 -enoyl-CoA isomerase

has potential value as a marker for various types of malignant cell types, and in fact may reflect fatty acid β -oxidation under these circumstances.

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