Halothane Metabolism: The Dihydrolipoamide Acetyltransferase Subunit of the Pyruvate Dehydrogenase Complex Molecularly Mimics Trifluoroacetyl–Protein Adducts[†]

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ABSTRACT: Monospecific antibodies (anti-CF$_3$CO antibodies), directed against trifluoroacetyl–protein adducts (CF$_3$CO–protein adducts) that are elicited in tissues of experimental animals and humans upon exposure to the anesthetic agent halothane, recognize cross-reactive proteins of 64 and 52 kDa in several tissues of rats and the liver of humans not previously exposed to the drug. These cross-reactive proteins mimic CF$_3$CO–protein adducts. Here, by the use of the anti-CF$_3$CO antibody as an immunoaffinity matrix, the protein of 64 kDa was purified from rat heart microsomal fractions. The amino acid sequence of six internal tryptic peptides exhibited 100% identity with the corresponding deduced amino acid sequences of the dihydrolipoamide acetyltransferase component (E2 subunit) of the rat liver pyruvate dehydrogenase (PDH) complex, as encoded by the cDNA clone pRMIT [Gershwin, M. E., Mackay, I. R., Sturgess, A., & Coppel, R. L. (1987) *J. Immunol. 138*, 3525–3531]. Lipoic acid, the prosthetic group of the E2 subunit of the PDH complex, exhibited immunochemical properties very similar to those of the hapten-derivative N^6-trifluoroacetyl-L-lysine (CF$_3$CO-Lys). On immunoblots, free lipoic acid inhibited the recognition of the E2 subunit, of the not yet identified protein of 52 kDa, and of the bulk of CF$_3$CO–protein adducts by anti-CF$_3$CO antibody with half-maximal inhibitory constants of 0.05, 10.0, and 8.5 mM, respectively. Lipoic acid also abolished the precipitation of the native E2 subunit by anti-CF$_3$CO antibody from solubilized rat heart mitochondrial fractions. These data suggest that lipoic acid is involved in the molecular mimicry of CF$_3$CO–protein adduct-related epitopes by the E2 subunit of the PDH complex.

The oxidative, cytochrome P450-dependent metabolism of the anesthetic agent halothane[1] (CF$_3$CHBrCl) leads to the formation of trifluoroacetyl–protein adducts (CF$_3$CO–protein adducts[2]) through covalent modification of lysine residues by the acyl halide intermediate CF$_3$COCl (Kenna et al., 1987, 1988b). CF$_3$CO–protein adducts have been detected on the surface of rat hepatocytes (Satoh et al., 1985a), in rat and rabbit liver microsomes (Satoh et al., 1985b; Kenna et al., 1988b), and in guinea pig centrilobular liver sections (Hubbard et al., 1989). Exposure of rats to structural analogues of halothane such as enflurane (Christ et al., 1989) or the candidate chlorofluorocarbon replacement 2,2-dichloro-1,1,1-trifluoroethane also leads to the formation of similar, immunochemically not discernible, CF$_3$CO–protein adducts, not only in the liver (Harris et al., 1991), but also in the kidney (Huwyler et al., 1992) and the heart (Huwyler & Gut, 1992). In the rat liver, CF$_3$CO–protein adducts have access

to Kupffer cells (Christen et al., 1991b), which are thought to be the resident cells in the liver competent for antigen presentation (Rubinstein et al., 1987).

Similar, if not identical CF$_3$CO–protein adducts are present in the livers of halothane-exposed human individuals (Kenna et al., 1988a; Pohl et al., 1989). Current evidence indicates that all individuals produce CF$_3$CO–protein adducts when exposed to halothane (Satoh et al., 1985a; Neuberger et al., 1987; Kenna et al., 1988a; Christen et al., 1991a). Halothane hepatitis occurs in about 1 in 30 000 and 1 in 3000 of patients, respectively, exposed to single and multiple doses of halothane and is thought to be due to an immune reaction toward such CF$_3$CO–protein adducts (Pohl et al., 1989). Since only sera from patients with halothane hepatitis contain antibodies against a group of liver CF$_3$CO–protein adducts ranging from 54 to 100 kDa (Kenna et al., 1987, 1988a), an immune reaction toward these CF$_3$CO–protein adducts appears to be restricted to a small subset of susceptible individuals, whereas the majority of individuals appear to immunochemically tolerate CF$_3$CO–protein adducts.

One of several possibilities for the lack of an immunological response of individuals toward CF$_3$CO–protein adducts might be the existence of natural immunological tolerance. Thus, based on a preexisting repertoire of self-peptides that structurally mimic epitopes present on CF$_3$CO–protein adducts, tolerance against CF$_3$CO–protein adduct-derived epitopes might have developed through thymic clonal deletion (Kappler et al., 1987, 1988; Kisielow et al., 1988; Ramsdell & Fowlkes, 1990) of maturing cells competent for the recognition of CF$_3$CO–protein adduct-derived epitopes. Alternatively, the induction of thymic and/or peripheral clonal anergy (Schwartz, 1990; Sprent et al., 1990) could render such cells silent toward

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[1] Abbreviations: halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane; PDH, pyruvate dehydrogenase; PBC, primary biliary cirrhosis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CF$_3$-CO-Lys, N^6-trifluoroacetyl-L-lysine; Lys(Ac), N^6-acetyl-L-lysine.

[2] The term CF$_3$CO–protein adduct designates any protein carrying trifluoroacetylated amino acid residues with no reference made to function and identity of that particular protein. The term anti-CF$_3$CO antibody designates the monospecific IgG fraction obtained from an antiserum directed against trifluoroacetylated rabbit serum albumin through affinity purification on a CF$_3$CO-Lys matrix (Christen et al., 1991a).

trifluoroacetylated motifs. An aberrant expression of such self-peptides might render individuals more susceptible for immunological reactions toward offending CF₃CO–protein adducts.

This novel concept has gained support by the identification of two constitutive proteins of 52 and 64 kDa, cross-reactive with anti-CF₃CO antibody, in the livers of rats (Christen et al., 1991a) and of human individuals (Gut et al., 1992) not exposed to halothane. Furthermore, the cross-reactive proteins of 52 and 64 kDa were found in high abundance in the heart and, to a much lesser degree, in the kidney, lung, spleen, thymus, and skeletal muscle of the rat (Christen et al., 1991a). Antibody exchange immunochemistry demonstrated that epitopes present on the proteins of 52 and 64 kDa of both the heart and the liver molecularly mimic CF₃CO–protein adduct-related epitopes, both in humans (Gut et al., 1992) and rats (Christen et al., 1991a). A role of these proteins in the susceptibility of individuals for halothane hepatitis was suggested by the fact that low levels or an absence of expression of the 52- and the 64-kDa proteins were found in liver biopsies of five of seven patients afflicted with the disease (Gut et al., 1992).

The identity of these proteins of 52 and 64 kDa as well as the molecular basis of the mimicry of CF₃CO–protein adduct-related epitopes was hitherto unknown. Here, using the anti-CF₃CO antibody as an immunoaffinity matrix, we have isolated the protein of 64 kDa, based on its high abundance in this tissue, from rat heart and identified it.

EXPERIMENTAL PROCEDURES

Materials. Aprotinin, leupeptin, pepstatin, soybean trypsin–chymotrypsin inhibitor, phenylmethanesulfonyl fluoride, taurocholic acid, (6RS)-lipoic acid (oxidized form), (6RS)-lipoic acid (reduced form), (6RS)-lipoamide, caprylic acid, and the PDH complexes of porcine heart and *Lactobacillus delbrückii* were all obtained from Sigma (St. Louis, MO). Goat anti-rabbit IgG (H+L) HRP-conjugate, Affi-Gel 102 amino terminal agarose, and the Affi-Gel Hz immunoaffinity kit were from Bio-Rad Laboratories (Richmond, CA). N^6-Trifluoroacetyl-L-lysine (CF₃CO-Lys) and N^6-acetyl-L-lysine (Lys(Ac)) were from Senn Chemicals (Dielsdorf, Switzerland). The enhanced chemiluminescence detection system was obtained from Amersham International (Amersham, U.K.).

Preparation of Rat Heart Subcellular Fractions. Male Sprague–Dawley rats (250–300 g) were sacrificed by decapitation. The hearts were removed, rinsed in ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.135 M NaCl, minced (5:1, vol/mass) in ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.135 M NaCl, 0.5 mM phenylmethanesulfonyl fluoride, and 60 μg/mL soybean trypsin–chymotrypsin inhibitor, and disrupted in a Potter Elvehjem homogenizer. The homogenate obtained was centrifuged at 4 °C at 3000g for 20 min to remove nuclei and cell debris. The supernatant fraction was sequentially centrifuged at 20000g for 20 min and at 105000g for 1 h, respectively, to obtain two fractions referred to as mitochondrial and microsomal fraction. The fractions were washed once in the above buffer and stored in aliquots at –80 °C until use. Note here that, based on immunoblot analysis (see below), the mitochondrial and the microsomal fraction obtained from rat hearts contained the proteins of 64 and 52 kDa that are cross-reactive with the monospecific anti-CF₃CO antibody (Christen et al., 1991a). Both fractions could be used interchangeably in immunopurification and/or immunoprecipitation experiments. No marker enzymes were measured to establish the purity of the respective fractions.

Gel Electrophoresis and Immunoblotting. Protein samples were mixed with equal volumes of dissociation buffer to give a final concentration of 12 mM Tris-HCl, pH 6.8, 8% SDS (w/v), 10% glycerol (v/v), and 40 mM dithiothreitol and were heated to 95 °C for 10 min. SDS–PAGE (Laemmli, 1970) was performed at room temperature with a Mini Protean II cell (Bio-Rad) using a 4.5% stacking and a 7.5% separating gel. Electrophoresis was for 40 min at 200 V. Proteins were transferred electrophoretically (Towbin et al., 1979) to nitrocellulose at 100 V × 1 h in 25 mM Tris, 192 mM glycine, and 20% methanol (v/v). After transfer, the nitrocellulose was stained with amido black, destained, and blocked for 2 h at room temperature with 10 mM Na₂HPO₄, 3 mM KH₂PO₄, 137 mM NaCl, pH 7.4, containing 2% (w/v) dry milk powder, and 0.02% (w/v) Thimerosal (subsequently termed the blocking solution). The nitrocellulose was cut into strips, incubated with anti-CF₃CO antibody (diluted 1:64) in blocking solution (500 μL) for 18 h at room temperature, washed (five times, 5 min each) with blocking solution, and incubated for 2 h at room temperature with goat anti-rabbit horseradish peroxidase-conjugated second antibody (diluted 1:250). Strips were washed (once for 5 min in blocking solution, four times for 7 min in 10 mM Na₂HPO₄, 3 mM KH₂PO₄, and 137 mM NaCl, pH 7.4, and once for 20 min in the same buffer), and CF₃CO–protein adducts were visualized by enhanced chemiluminescence detection. Where indicated, incubation of polypeptides blotted onto nitrocellulose with the anti-CF₃CO antibody was done in the presence of effectors. For analysis of immunoblots, the films obtained after exposure of immunoblots to the enhanced chemiluminescence system were scanned using a computing densitometer (Molecular Dynamics 300A) operated with Molecular Dynamics Image Quant v3.0 software. In order to compare the signals obtained on different immunoblots of a particular set of experiments, the signals generated from the corresponding control incubations (i.e., the proteins of 64 and 52 kDa or where appropriate the bulk of CF₃CO–protein adducts included on each individual immunoblot as internal standards) were arbitrarily set as 100% response; these signals were kept within 80% of the maximal dynamic range of the films (Hyperfilm ECL, Amersham International) used.

Preparation of Anti-CF₃CO Antibody. The monospecific anti-CF₃CO antibody used in these experiments was prepared as described (Christen et al., 1991a). Briefly, the IgG fraction obtained from a polyclonal antiserum raised in rabbits against trifluoroacetylated rabbit serum albumin was applied to an Affi-Gel 102 matrix to which the hapten-derivative CF₃CO-Lys had been coupled. After being extensively washed, the monospecific anti-CF₃CO antibody was then displaced from the affinity matrix by the addition of 50 mM CF₃CO-Lys to the elution buffer. After an extensive dialysis, aliquots of the final preparation of anti-CF₃CO antibody were stored at –80 °C (0.1 mg IgG/mL) and thawed only once.

Immunoaffinity Purification of 64-kDa Protein. Aliquots of the rat heart microsomal fraction (10 mg protein/mL, 300 mg of protein total) were solubilized at 4 °C in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5 mM phenylmethanesulfonyl fluoride, 60 μg/mL soybean trypsin–chymotrypsin inhibitor, 20 μg/mL aprotinin, 0.7 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 10 mg/mL taurocholic acid for 90 min under constant shaking. After centrifugation (105000g, 1 h), the pellet was discarded, and the supernatant (30 mL, 140 mg of protein) was recirculated (10 mL/h) at 4 °C for 16 h over an anti-CF₃CO antibody immunoaffinity column (~2 mL bed volume), preequilibrated with 50 mM

Tris-HCl, pH 7.4, 0.5 M NaCl, and 10 mg/mL taurocholic acid. The immunoaffiniy matrix had been prepared by coupling anti-CF$_3$CO antibody to Affi-Gel Hz (Bio-Rad, ~1.2 mg IgG coupled/mL of gel). After sample application, the immunoaffinity column was washed (10 bed volumes) with 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 10 mg/mL taurocholic acid, until protein elution (280 nm) reached baseline level. To displace the bound protein, one bed volume of the same buffer containing 100 mM CF$_3$CO-Lys, was applied. After incubation for 16 h, displaced proteins were eluted in the same buffer. Fractions (1 mL) reactive with anti-CF$_3$CO antibody were identified by an ELISA assay (Christen et al., 1991a) and analyzed by SDS–PAGE and immunoblotting.

Immunoprecipitation of 64-kDa Protein. A rat heart mitochondrial fraction (10 mg/mL) was solubilized at 4 °C in 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5 mM phenylmethanesulfonyl fluoride, 60 μg/mL soybean trypsin–chymotrypsin inhibitor, 20 μg/mL aprotinin, 0.7 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 10 mg/mL taurocholic acid for 90 min. After centrifugation at 105000g for 1 h, aliquots (100 μL) of the supernatant were incubated, in the presence of the indicated effectors, for 16 h under constant shaking with 50 μL of Affi-Gel Hz gel to which anti-CF$_3$CO antibody had been coupled (~1.2 mg IgG/mL gel). After the gel was washed with 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 10 mg/mL taurocholic acid (five times, 1 mL each), proteins were displaced by incubation with 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 10 mg/mL taurocholic acid, and 100 mM CF$_3$CO-Lys (100 μL) for 16 h. After centrifugation, aliquots of the supernatants (25 μL) were analyzed at room temperature by SDS–PAGE and immunoblotting for the presence of the 64-kDa protein. Aliquots (25 μL) of the solubilizates remaining after their incubation with anti-CF$_3$CO antibody were analyzed similarly.

Amino Acid Sequence Analysis. For N-terminal amino acid sequence analysis, approximately 10 μg of the purified 64-kDa protein was subjected to SDS–PAGE, electrophoretically transferred to a PVDF-membrane, stained with Coomassie Blue, excised, and applied to a pulsed liquid-phase sequencer 477A (Applied Biosystems) connected to a 120A PTH-amino acid analyzer (Applied Biosystems). Purified 64-kDa protein (~40 μg) was denatured in 8 M urea in 0.1 M NH$_4$HCO$_3$, reduced with 4 mM dithiothreitol (1 h at 37 °C), alkylated with 10 mM iodoacetamide (15 min at 37 °C in the dark), and digested with trypsin at 37 °C for 16 h. One aliquot of the sample was centrifuged at 10000g, and soluble fragments in the supernatant were separated by RP-HPLC on a C$_8$ Aquapore RP-300 column (2.1 × 250 mm) (Stone & Williams, 1988). Twelve fragments (S1–S12) were collected in Eppendorf tubes and stored at –80 °C until use. The other aliquot was adjusted to 6 M in guanidine hydrochloride, and tryptic fragments were separated on RP-HPLC without prior centrifugation under the conditions described above. The fragments G1–G19 were collected and stored at –80 °C until amino acid sequence analysis.

RESULTS

Immunoaffinity Purification of Protein of 64-kDa. In rats not treated with halothane, the constitutive proteins of 64 and 52 kDa, cross-reactive with anti-CF$_3$CO antibody, are highly abundant in heart tissue (Christen et al., 1991a) and found to be associated with the mitochondrial and microsomal fractions thereof. Although both proteins were readily recognized by anti-CF$_3$CO antibody on immunoblots, pre-
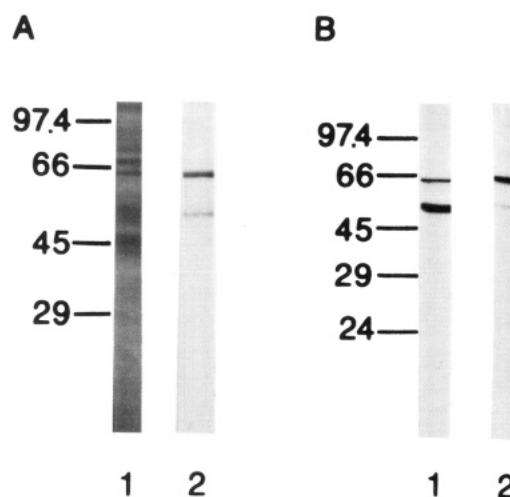

FIGURE 1: Purification of the 64-kDa cross-reactive protein. Rat heart microsomal fractions were solubilized and subjected to immunoaffinity chromatography on a matrix containing anti-CF$_3$-CO antibody coupled to Affi-Gel Hz as described in Experimental Procedures. (A) Silver staining of gels after SDS–PAGE. Lane 1, 100 μg protein/cm slot width of solubilized rat heart microsomal fraction; lane 2, 100 ng of purified 64-kDa protein after immunoaffinity chromatography. (B) The corresponding immunoblots were developed with anti-CF$_3$CO antibody. Lane 1, rat heart microsomal fraction; lane 2, purified 64-kDa protein after immunoaffinity chromatography. Migration distances of proteins of known molecular mass (in kilodalton) are indicated.

liminary experiments revealed that the anti-CF$_3$CO antibody was capable of immunoprecipitating only the 64-kDa protein from solubilized rat heart mitochondrial and microsomal fractions (not shown); a negligible degree (typically ≤1%) of coprecipitation of the 52-kDa protein was observed. Consequently, a single-step purification strategy involving immunoaffinity chromatography was developed that led reproducibly (n > 5) to the purification of the 64-kDa protein from the supernatant (105000g) of solubilized rat heart microsomal fractions (Figure 1A, lane 2; typical yield ~0.02% of the total protein present in the supernatant). The purified protein of 64 kDa retained its reactivity toward anti-CF$_3$CO antibody on immunoblots (Figure 1B, lane 2) and lacked any significant contamination by the 52-kDa protein (Figure 1B, lane 2).

Identification of Protein of 64 kDa. The amino acid sequences of six internal peptides obtained from the purified 64-kDa protein were found to be highly homologous with regions of the deduced amino acid sequences of the rat and human liver E2 subunits of the PDH complex, respectively, as encoded by the clones pRMIT (Gershwin et. al., 1987) and pHuMIT (Coppel et al., 1988). Thus, 100% identity with the corresponding deduced amino acid sequences encoded by pRMIT was found for the internal peptides 1–6 (Figure 2). Except for peptide 1 (60% identity in 15 amino acids) and peptide 5 (94% identity in 17 amino acids), 100% identity with the corresponding deduced amino acid sequences encoded by pHuMIT was also found.

Since pRMIT codes for a portion of the rat E2 subunit lacking the N-terminus (Gershwin et al., 1987), a correlation with the N-terminal amino acid sequence obtained from the purified 64-kDa protein was not possible. However, the N-terminal amino acid sequence of the 64-kDa protein (Figure 2) exhibited about 71% homology to a corresponding deduced amino acid sequence encoded by pHuMIT (i.e., residues 54–67), which is not the N-terminus of the human E2 subunit protein (Coppel et. al., 1988). Moreover, the 64-kDa protein exhibited 64% N-terminal homology (in 14 amino acids) with bovine E2$_{LB}$ (Rahmatullah et al., 1989a). Note that, in

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64-kDa protein  (NH -terminus)    01   SLPPGQKVPLIPLV    14
pHuMIT                            54   ----H-----PS-S    67
Bovine heart E2_LB                01   ----HE----PS-S    14

64-kDa protein  (Peptide 1)            AAPAAAAAAPP.GPR
pRMIT                            221   -----------.---  233
pHuMIT                          364   V---P--VV--T--G  377
27-kDa E2₁fragment                     ---TP---V--.PSP

64-kDa protein  (Peptide 2)            ILVPEGTR
pRMIT                           103   --------  110
pHuMIT                          242   --------  249

64-kDa protein  (Peptide 3)            QNHVVDVSVAVSTPAGLITP
pRMIT                           328   --------------------  347
pHuMIT                          471   --------------------  490

64-kDa protein  (Peptide 4)            DVPLGTPLCIIVEK
pRMIT                           111   --------------  124
pHuMIT                          250   --------------  263

64-kDa protein  (Peptide 5)            GFDVASVMSVTLSCDHR
pRMIT                           428   -----------------  444
pHuMIT                          571   ------M----------  587

64-kDa protein  (Peptide 6)            VVDGAVGAQWLA
pRMIT (COOH-terminus)           445   ------------  456
pHuMIT                          588   ------------  599
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FIGURE 2: Amino acid sequence comparison. The N-terminal amino acid sequence was obtained from the intact 64-kDa protein. Tryptic fragments of the 64-kDa protein were separated by RP-HPLC, yielding fragments S1–S12 and G1–G19 (see Experimental Procedures). From the selected fragments S1, S2, and G18, the sequences corresponding to peptide 1, 2, and 3 were obtained. Fragment G12 yielded a triple sequence which could be assigned to the peptides 4, 5, and 6. The sequences were compared, where available, with the amino acid sequence of bovine heart E2_LB or the 27-kDa E2₁ fragment (Rahmatullah et al., 1989a) and the deduced amino acid sequences of clones pHuMIT (Coppel et al., 1988) and pRMIT (Gershwin et al., 1987), respectively, covering the complete human and a portion of the rat E2 subunit of the PDH complex. (–) indicates identical residues and (·) indicates gaps that were introduced (Coppel et al., 1988)

contrast to the N-terminus of the 64-kDa protein from rat heart (this study), the N-terminus of bovine E2_LB was unveiled by tryptic treatment of the authentic E2 subunit only, whose N-terminus was blocked (Rahmatullah et al., 1989a). On the basis of these data, we conclude that the protein of 64 kDa, purified from solubilized rat heart microsomal fractions by anti-CF₃CO antibody immunoaffinity chromatography, is identical with the E2 subunit of the rat liver PDH complex (Gershwin et al., 1987). It is noteworthy that the recognition by anti-CF₃CO antibody is not restricted to the E2 subunit of the rat heart PDH complex, but it is also found with the corresponding E2 subunits of porcine heart and of *Lactobacillus delbrückii* (U. Christen and J. Gut, unpublished observation).

Lipoic Acid Inhibits Recognition of the E2 subunit of the PDH Complex. The attempt to map the site(s) recognized by anti-CF₃CO antibody within the E2 subunit was unsuccessful because none of the tryptic fragments G1–G19 showed cross-reactivity with the anti-CF₃CO antibody in ELISA-based analysis (data not shown). This finding could be due to the destruction of the antigenic determinant(s) in the process of denaturation of the purified E2 subunit prior to its tryptic digestion (see Experimental Procedures) or, alternatively, by the tryptic cleavage process itself.

Within the E2 subunit, lipoic acid is covalently bound to the N^6-amino group of the single lysine present in a consensus motif (ETDKA) that is found in rat (Gershwin et al., 1987), human (Coppel et al., 1988) and bovine (Spencer et al., 1984) E2 subunit amino acid sequences. We speculated that lipoic acid might bear structural resemblance to the hapten derivative
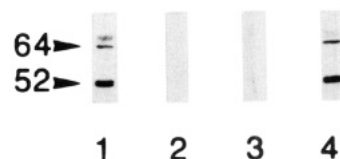

FIGURE 3: Inhibition of recognition of 64- and 52-kDa proteins on immunoblots. Rat heart mitochondrial fractions (100 μg protein/cm slot width) were analyzed by SDS–PAGE and immunoblotting. The indicated effectors were included in the incubations of the immunoblots with anti-CF₃CO antibody. Lane 1, control experiment, no addition; lane 2, 50 mM CF₃CO-Lys; lane 3, 100 mM lipoic acid; lane 4, 100 mM trifluoroacetic acid. Arrowheads indicate the migration positions of the 64- and the 52-kDa proteins.
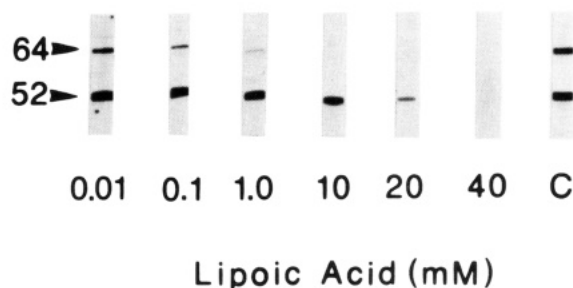

Lipoic Acid (mM)

FIGURE 4: Concentration-dependent inhibition by lipoic acid of the recognition of the 64- and the 52-kDa protein. Rat heart mitochondrial fractions (100 μg/cm slot width) were analyzed by SDS–PAGE and immunoblotting. Increasing concentrations of lipoic acid were included in the incubations of the immunoblots with anti-CF₃-CO antibody. C indicates the control experiment in the absence of lipoic acid. Arrowheads indicate the migration positions of the 64- and the 52-kDa proteins. In order to estimate the apparent half-maximal inhibitory constants IC₅₀, the films obtains after exposure of the immunoblots to the enhanced chemiluminescence system were scanned using a computing densitometer (Molecular Dynamics 300A).

Table I: Apparent Half-Maximal Inhibitory Constants IC_{50} As Determined by Competitive Immunoblotting

target	inhibitor (mM)	
	lipoic acid	CF₃CO-Lys
E2 subunit	0.05	0.01
protein of 52 kDa	10.0	0.02
bulk of CF₃CO–protein adducts	8.5	0.3
A[a]	7.0	nd
B[a]	2.8	nd
C[a]	>40.0	nd
D[a]	11.0	nd

[a] Randomly selected CF₃CO–protein adducts as indicated in Figure 6; nd, not determined.

CF₃CO-Lys. Hence, we tested if lipoic acid[3] could be involved in the molecular mimicry of CF₃CO–protein adducts by the E2 subunit of the PDH complex. In fact, free lipoic acid inhibited the recognition of the E2 subunit by anti-CF₃CO antibody on immunoblots of rat heart mitochondrial fractions (Figure 3, lane 3). The inhibition was concentration-dependent (Figure 4) with an apparent IC₅₀ value of 0.05 mM (Table I). Interestingly, the recognition of the cross-reactive protein of 52 kDa protein was also sensitive to lipoic acid (Figure 3, lane 3) in a concentration-dependent manner with an apparent IC₅₀ value of 10 mM (Table I). Thus, lipoic acid exhibits properties similar to those of the hapten derivative CF₃CO-Lys. The latter is a potent inhibitor for the recognition of both the E2 subunit of the PDH complex and the not yet identified protein of 52 kDa (Figure 3, lane 2), respectively, with apparent IC₅₀ values of 0.01 and 0.02 mM (Table I). As

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[3] Except where noted, the oxidized form of racemic (6RS)-lipoic acid was used.

Table II: Inhibition of Recognition of E2 Subunit of Rat Heart PDH Complex by Anti-CF$_3$CO Antibody

inhibitor	apparent IC$_{50}$ (mM)
(6RS)-lipoic acid (oxidized form)	0.05
(6RS)-lipoamide	0.08
(6RS)-lipoic acid (reduced form)[a]	0.33
caprylic acid	16.0
CF$_3$CO-Lys	0.01
Lys(Ac)	0.73
trifluoroacetic acid	no inhibition at 100 mM
L-lysine	no inhibition at 100 mM

[a] Highest concentration of lipoic acid used was 10 mM; the competitive immunoblotting experiment was performed in the presence of 10 mM dithiothreitol.

shown in Table II, the reduced form of lipoic acid is less efficient (IC$_{50}$ = 0.33 mM) in competing with anti-CF$_3$CO antibody binding to the E2 subunit; the lack of bulky substitutions in positions 6 and 8 of the carbon backbone of caprylic acid further increases the IC$_{50}$ to 16 mM. When compared with CF$_3$CO-Lys (IC$_{50}$ = 0.01 mM), an increased IC$_{50}$ is also observed with Lys(Ac) (0.73 mM) (Table II), while the incomplete hapten derivatives L-lysine and trifluoroacetic acid do not interfere with the recognition of the E2 subunit by anti-CF$_3$CO antibodies (Table II).

A general inhibition of antibody binding to the corresponding antigens on immunoblots through the presence of hapten derivatives (i.e., CF$_3$CO-Lys) or mimics thereof (i.e., lipoic acid) was excluded in that in separate control experiments, no inhibition of recognition of microsomal epoxide hydrolase by anti-epoxide hydrolase antibody was observed on immunoblots of rat liver microsomal membranes in the presence of 100 mM CF$_3$CO-Lys or 100 mM lipoic acid (not shown). Also, incomplete hapten derivatives such as trifluoroacetic acid (100 mM; Figure 3, lane 4) or lysine (100 mM; data not shown) did not interfere with the extent of recognition of both the E2 subunit of the PDH complex or the cross-reactive protein of 52 kDa.

Lipoic Acid Inhibits the Immunoprecipitation of the E2 Subunit of the PDH Complex. The inhibitory properties of lipoic acid in the recognition of the E2 subunit by anti-CF$_3$-CO antibody were not limited to epitopes of the denatured E2 subunit as present on immunoblots. Anti-CF$_3$CO antibody very specifically bound the native E2 subunit of the PDH complex of the solubilized rat heart microsomal fraction (Figure 1B, lane 2) and precipitated it from the mitochondrial fraction (Figure 5, lane 2A). No 52-kDa protein was precipitated under these conditions. At 50 mM each, CF$_3$-CO-Lys (Figure 5, lane 3A) and lipoic acid (Figure 5, lane 4A), but not trifluoroacetic acid (Figure 5, lane 5A), abolished the precipitation of the native E2 subunit by anti-CF$_3$CO antibody from rat heart mitochondrial fractions. As shown in Figure 5, lanes 3B, 4B, and 5B, respectively, the presence of CF$_3$CO-Lys, lipoic acid, and trifluoroacetic acid did not interfere with the integrity of the epitopes recognized by anti-CF$_3$CO antibody on either the E2 subunit or the 52-kDa protein. The implication of the data is twofold. First, lipoic acid is a mimic of the hapten derivate CF$_3$CO-Lys by its ability to abolish the recognition by anti-CF$_3$CO antibody of the respective epitope(s) in both the solubilized (i.e., native) and immunoblotted (i.e., denatured) state of the E2 subunit of the PDH complex. Second, in contrast to the epitope(s) of the E2 subunit, the epitope(s) on the 52-kDa protein can be recognized by anti-CF$_3$CO antibody solely in the denatured state of that protein.
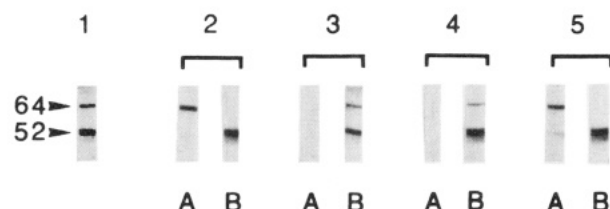

FIGURE 5: Immunoprecipitation of the native 64-kDa protein by anti-CF$_3$CO antibody. Rat heart mitochondrial fractions (25 μg of protein) were solubilized and incubated with anti-CF$_3$CO antibody coupled to Affi-Gel Hz in the absence (lane 2) or the presence of 50 mM CF$_3$CO-Lys (lane 3), 100 mM lipoic acid (lane 4), and 100 mM trifluoroacetic acid (lane 5). Aliquots (25 μL/slot) of precipitated proteins after their displacement by 100 mM CF$_3$CO-Lys (A) and of the solubilizate remaining after incubation with the anti-CF$_3$CO antibody matrix (B) were analyzed by SDS–PAGE and immunoblotting (see Experimental Procedures). Lane 1, 25-μL aliquot of unprecipitated solubilized rat heart mitochondrial fraction. Arrowheads indicate the migration positions of the 64- and the 52-kDa proteins.

Lipoic Acid Inhibits the Recognition of CF$_3$CO–Protein Adducts. CF$_3$CO–protein adducts of distinct molecular mass, recognized by anti-CF$_3$CO antibody on immunoblots (Figure 6A, lane 1), are most abundant in the liver of rats exposed to halothane but are also found to a lesser extent in the kidney (Huwyler et al., 1992), the heart (Huwyler & Gut, 1992), and the testis (Kenna et al., 1991) of these animals. Based on previous experiments in this laboratory (Christen et al., 1991a), the recognition of CF$_3$CO–protein adducts by anti-CF$_3$CO antibody was abolished in presence of 50 mM CF$_3$-CO-Lys (Figure 6A, lane 2) and not affected by the presence of 100 mM trifluoroacetic acid (Figure 6A, lane 3). Similarly to CF$_3$CO-Lys [mean apparent IC$_{50}$ = 0.3 mM (Table I)], lipoic acid inhibited in a concentration-dependent manner (Figure 6B, lanes 1–5) the recognition of the bulk of CF$_3$-CO–protein adducts by anti-CF$_3$CO antibody with an apparent mean IC$_{50}$ value of 8.5 mM (Table I). For discrete CF$_3$-CO–protein adducts, clear differences in the apparent IC$_{50}$ values became evident. Thus, for the randomly selected CF$_3$-CO–protein adducts A–D (see Figure 6B), apparent IC$_{50}$ values of 7.0, 2,8, >40, and 11.0 mM, respectively, were determined (Table I). These data suggest differences in the degree of the structural relatedness of epitopes present on discrete CF$_3$CO–protein adducts that can be explored by the use of hapten derivatives (CF$_3$CO-Lys) or mimics thereof (such as lipoic acid).

DISCUSSION

The identity of the constitutively expressed proteins of 52 and 64 kDa, which mimic CF$_3$CO–protein adduct-related epitopes and which are expressed in several tissues of the rat and in human liver (Christen et al., 1991a), as well as the molecular basis of this mimicry, was unknown to date. In this report, we have identified the protein of 64 kDa as the E2 subunit of the PDH complex based on the sequence homology between selected internal peptides of the protein of 64 kDa and regions of the deduced amino acid sequences of the rat and human E2 subunits of the PDH complexes, respectively, encoded by the clones pRMIT (Gershwin et. al., 1987) and pHuMIT (Coppel et al., 1988). A number of immunochemical experiments indicate that lipoic acid is involved in the molecular mimicry of CF$_3$CO–protein adduct-related epitopes for several reasons: First, on immunoblots, the recognition of the purified E2 subunit by anti-CF$_3$CO antibody is abolished in the presence of either lipoic acid (apparent IC$_{50}$ = 0.05 mM) or the hapten derivative CF$_3$CO-Lys (apparent IC$_{50}$ =
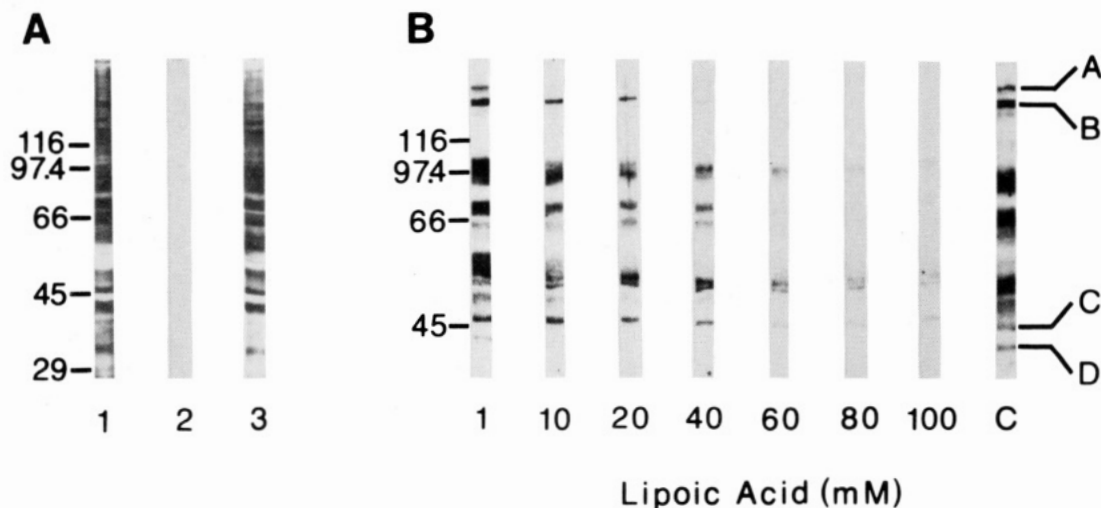
FIGURE 6: Inhibition of the recognition by anti-CF₃CO antibody of CF₃CO–protein adducts. Rat liver homogenates were obtained after pretreatment of rats with a single dose of halothane (10 mmol/kg of body mass, intraperitoneally) and analyzed by SDS–PAGE and immunoblotting (Christen et al., 1991a). Effectors as indicated were included in the incubations of the immunoblots with anti-CF₃CO antibody. (A) Lane 1, no effector; lane 2, 50 mM CF₃CO-Lys was included; lane 3, 100 mM trifluoroacetic acid was included. (B) Lipoic acid was included in a concentration-dependent manner. C indicates the control experiment in the absence of lipoic acid. In order to estimate the apparent half-maximal inhibitory constants IC$_{50}$ of the bulk of the CF₃CO–protein addcuts or the randomly selected CF₃CO–protein adducts A–D, the films obtained after exposure of the immunoblots to the enhanced chemiluminescence system were scanned using a computing densitometer (Molecular Dynamics 300A). Migration distances of proteins of known molecular mass (in kilodaltons) are indicated.

0.01 mM). Second, the immunoprecipitation of the native E2 subunit by anti-CF₃CO antibody from solubilized rat heart mitochondrial fractions is inhibited in presence of either lipoic acid or CF₃CO-Lys. Third, similar to CF₃CO-Lys (apparent IC$_{50}$ = 0.3 mM), lipoic acid inhibits (apparent IC$_{50}$ = 8.5 mM) the recognition of the bulk of CF₃CO–protein adducts by anti-CF₃CO antibody on immunoblots of livers of halothane-exposed rats.

The major adduct formed on proteins upon hepatic metabolism of halothane has been identified as N^6-trifluoroacetyl-L-lysine by ^{19}F nuclear magnetic resonance (NMR) spectroscopy (Harris et al., 1991). The data in this report suggest that free lipoic acid per se is structurally sufficiently close to immunochemically mimic these trifluoroacetylated motifs present on CF₃CO–protein adducts, and circumstantial evidence might imply that this is also the case when lipoic acid is covalently attached to the corresponding lipoyl-binding site-(s) of the E2 subunit. Thus, in earlier antibody exchange experiments (Christen et al., 1991a), anti-CF₃CO antibody spontaneously exchanged on immunoblots from the intact rat heart E2 subunit of the PDH complex (i.e., the protein of 64 kDa) to target CF₃CO–protein adducts present in the livers of halothane-exposed rats. Similarly, spontaneous exchange of anti-CF₃CO antibodies from the cross-reactive protein of 64 kDa of human liver to CF₃CO–protein adducts present in the liver of human individuals exposed to halothane (Gut et al., 1992) could be demonstrated. This transfer was sensitive to competition by CF₃CO-Lys which, in the current study, has been shown to exhibit inhibitory properties identical to those of free lipoic acid. However, although the hapten derivative CF₃CO-Lys and its mimic lipoic acid are common denominators of potent inhibition of the recognition by anti-CF₃CO antibody of the structurally closely related epitopes present on either the E2 subunit, the protein of 52 kDa, or on CF₃CO–protein adducts, it can not be excluded that some epitope other than the lipoyl-binding domain(s) of the E2 subunit might be involved. Although the clone pRMIT does not code for the complete rat liver E2 subunit protein (Gershwin et. al., 1987), it appears now that the rat heart E2 subunit of the PDH complex has two lipoyl-binding domains (Matuda et al., 1992) presumed to interact with anti-CF₃CO antibody.

Thus, current experiments are aimed at the isolation of the intact native epitope [i.e., the presumed lipoyl-binding site(s) of the rat heart E2 subunit of the PDH complex] in order to determine the number of epitopes of the E2 subunit protein that are reactive with anti-CF₃CO antibody and to directly assess the structural relatedness to cross-reactive epitopes present on CF₃CO–protein adducts. Furthermore, studies are in progress to isolate and identify the cross-reactive protein-(s) of 52 kDa. Based on the immunochemical properties that are similar to those of the E2 subunit of the PDH complex, it is tempting to speculate that the protein(s) of 52 kDa might correspond to the E2 subunits of the branched chain ketoacid dehydrogenase complex, the oxoglutarate dehydrogenase complex, and/or protein X (Yeaman, 1986; Lau et al., 1988; Rahmatullah et al., 1989b; Perham, 1991).

The closely related mitochondrial E2 subunits of the PDH complex, the branched chain ketoacid dehydrogenase complex, the oxoglutarate dehydrogenase complex, and protein X, a constituent of the PDH complex, have recently been identified as the major autoantigens of primary biliary cirrhosis (PBC) (Coppel et al., 1988; Van de Water et al., 1988a; Fussey et al., 1988, 1989; Fregeau et al., 1990; Surh et al., 1989). A striking structural similarity among these autoantigens is the presence of one or more lipoyl-binding domains to which lipoic acid is covalently attached (Perham, 1991; Yeaman, 1986). A number of studies have indicated that the lipoylated domains of these autoantigens are immunodominant epitopes in that sera of human individuals afflicted with primary biliary cirrhosis preferentially recognize such domains (Surh et al., 1989; Leung et al., 1990; Van de Water et al., 1988b). The isolation, in the present study, of one of these autoantigens, namely, the E2 subunit of the PDH complex, by the use of a monospecific anti-CF₃CO antibody, which is directed against trifluoroacetylated motifs on CF₃CO–protein adducts, does imply a close structural relatedness between epitopes on CF₃-CO–protein adducts, elicited upon exposure of individuals to halothane, and the immunodominant epitopes associated with PBC. In this context, it might be noteworthy that some antisera of patients afflicted with PBC tend to cross-react with trifluoroacetylated rabbit serum albumin or with trifluoroacetylated rat liver microsomal proteins of 100, 76, and

57 kDa, used as model antigens to establish a diagnosis of halothane hepatitis in human individuals (Martin et al., 1988, 1990), although a bearing of this observation in the etiology of either halothane hepatitis or PBC remains obscure at present.

The competitive immunoblot analysis performed in this study suggests that lipoic acid structurally mimics the hapten-derivative CF_3CO-Lys which itself mimics the trifluoroacetylated motifs present on CF_3CO–protein adducts; circumstantial evidence suggests that lipoic acid covalently attached to the lipoyl-binding domain(s) of the E2 subunit of the PDH complex could exhibit similar properties. Mimicry of antigenic determinants by structures closely related to lipoic acid might not be so uncommon. Thus, octanoylation of the E2 subunits of the PDH complex and the 2-oxoglutarate dehydrogenase complex occurs in the lipoic acid biosynthesis mutant *Escherichia coli* strain JRG26 under lipoic acid deprivation (Ali et al., 1990). Sera of patients with PBC recognized these variant E2 subunits similarly to the lipoylated E2 subunit counterparts (Fussey et al., 1990); E2 subunits lacking either octanoyl- or lipoyl-modifications were not antigenic. These data suggest that upon replacing lipoic acid by octanoic acid, a mimicry of the unique peptide-cofactor conformation of the authentic determinant of the lipoyl-binding domain was created.

It remains to be elucidated if the occurrence of epitopes that partially or fully mimic trifluoroacetylated motifs in the repertoire of self-peptides might help to establish immunological tolerance of individuals toward CF_3CO–protein adducts. Furthermore, since a portion of patients afflicted with halothane hepatitis exhibits aberrant low levels of immunochemically recognizable E2 subunit protein and 52-kDa protein (Gut et al., 1992), the question arises if such irregularities might increase the risk of human individuals for the development of halothane hepatitis. The identification of such susceptibility factors might become increasingly important, since exposure to the clinical replacements of halothane, enflurane, and isoflurane (Christ et al., 1989) as well as to candidate replacements of ozone-depleting chlorofluorocarbons (Manzer, 1990) such as 2,2-dichloro-1,1,1-trifluoroethane leads to formation of potentially immunogenic protein adducts (Harris et al., 1991; Huwyler et al., 1992) that are structurally very similar to those elicited upon exposure of individuals to halothane.

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