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Immunochemical evidence for an inactive form of cytochrome oxidase in mitochondrial membranes of ethanol-fed rats

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Previous studies have established that rats fed ethanol chronically exhibit a 50% decrease in hepatic mitochondrial cytochrome oxidase compared to pair-fed controls, based on both heme aa_3 content and specific activity. To determine whether the 'missing' 50% of cytochrome oxidase is present in the membrane but catalytically inactive, or entirely absent, we used immunochemical techniques to determine the content of cytochrome oxidase protein in hepatic submitochondrial particles. Rabbit antiserum against purified rat liver cytochrome oxidase precipitated cytochrome oxidase from detergent-solubilized submitochondrial particles. Immunoinhibition titrations of a fixed amount of anti-oxidase serum with increasing amounts of submitochondrial particle protein showed that similar percentages of added oxidase activity were recovered in supernatants after immunoprecipitation with preparations from both alcoholic and control rats. Similarly, titrations of a fixed amount of submitochondrial particle protein with increasing amounts of antiserum showed comparable decreases in oxidase activity. Equivalent amounts of protein were obtained in immunoprecipitates from both preparations. Immunoprecipitates demonstrated comparable oxidase subunit profiles by electrophoresis, except that one additional band, migrating in the region of oxidase subunit IV, was present in samples from alcoholic rats. The data indicate that cytochrome oxidase immunologic reactivity is quantitatively similar in both types of membranes. The results suggest that the 'missing' cytochrome oxidase is actually present within the membranes of alcoholic animals in an inactive form, apparently devoid of heme aa_3.

Introduction

We have previously shown that rats fed ethanol chronically have decreased amounts of hepatic mitochondrial cytochrome oxidase [1,2], cytochromes *b* [2,3], NADH-dehydrogenase [4] and ATPase [1] compared to pair-fed control rats. These selective alterations in membrane proteins are accompanied by modifications in membrane-lipid composition and physical properties [5,6], and account for the lower rates of respiration and ATP synthesis found in vitro after chronic ethanol treatment [1,2,7]. These effects of ethanol consumption have been established by biochemical criteria, such as heme reduction and enzyme specific activity. While such approaches have clearly demonstrated decreased amounts of enzymatically active (functional) proteins, it is important to recognize that the physical contents of the particular protein or apoprotein moieties, per se, have not been determined in these previous studies. In particular, the fate of the proteins apparently 'missing' from

Abbreviations: P_i, inorganic phosphate; Cyt, cytochrome.

the membranes of alcoholic animals has not been addressed.

The decline of mitochondrial cytochrome oxidase content after ethanol feeding has been recognized for many years and reported widely [1,2,8–11]. Our own studies utilizing absorbance-difference spectroscopy with liver mitochondria, submitochondrial particles and isolated hepatocytes indicated that rats fed ethanol chronically have only 50–60% as much cytochrome oxidase per mg membrane protein as control rats, based on heme aa_3 absorbance [2]. Kinetic properties of the oxidase remaining in the membrane appeared unchanged, and the oxidase interacted with all of the cytochrome *c* [2,12]. Thus, we asked whether the ethanol-induced decrease in cytochrome oxidase was due to the presence of only one-half the normal amount of fully functional oxidase or to a mixture of active and inactive forms of cytochrome oxidase. To answer this question, we have used immunochemical techniques to measure the amount of cytochrome oxidase protein in membrane preparations. Our approach was first to purify cytochrome oxidase from control rats, use the protein to raise a specific antibody in rabbits, and then employ the antiserum as a probe for detecting the antigen (cytochrome oxidase protein) in submitochondrial particles from alcoholic and control rats.

Experimental procedures

Animals. Male, Sprague-Dawley, littermate rats (Charles River Breeding Laboratories) were pair-fed a totally liquid diet (Bio-Serv, Inc., Frenchtown, NJ) containing ethanol as 36% of total joules [13] for 40–45 days as previously described [1]. Similar rats fed a standard chow diet and water ad libidum were used for isolation of cytochrome oxidase.

Preparations. Rat liver submitochondrial particles were prepared from freshly isolated mitochondria by sonication of mitoplasts in a medium containing $MgCl_2$, ATP and substrates, as previously described [1], and stored at −20°C. Protein concentration of submitochondrial particles was determined by the cyanide biuret procedure [14] using bovine serum albumin as a standard. Heme aa_3 contents, determined spectrophotometrically, of the preparations used for the experiments reported in this paper were: control, 0.41 ± 0.03; alcoholic, 0.25 ± 0.02 nmol/mg protein ($n = 7$; $p < 0.01$), comparable to those found previously [2].

Rat liver cytochrome oxidase was isolated from submitochondrial particles by cholate and ammonium sulfate fractionations according to the protocol described by Kuboyama et al. [15] for bovine heart. Fractions were monitored by absorbance difference spectroscopy with an Aminco DW-2A spectrophotometer at room temperature. An absorptivity coefficient of $E_{605\ nm} = 12.0$ $mM^{-1} \cdot cm^{-1}$ (dithionite-reduced minus oxidized) was used to determine heme *a* concentration [15]. Protein concentration of purified oxidase preparations was measured by the Lowry method using bovine serum albumin as a standard. The oxidase preparation used for antibody production had a heme *a*/protein ratio of 7.3, and appeared free of other cytochromes by spectral analysis. Purified oxidase was suspended in 0.1 M NaP_i, 0.1% Tween-80 (pH 7.4), at 16 mg protein/ml, and stored in aliquots at −70°C. In a typical preparation, a yield of 31 mg of oxidase was obtained from 600 mg of submitochondrial particles.

Antibody production. For immunization, rat liver cytochrome oxidase was diluted to 5.5 mg/ml in 50 mM NaP_i, pH 7.4, and mixed with an equal volume of Freund's complete adjuvant. New Zealand white rabbits (3 kg) received 1 ml of emulsion (2.7 mg oxidase) subcutaneously, distributed over multiple sites on the back. Rabbits were boosted at biweekly intervals with 0.7 mg oxidase, diluted as above, in Freund's incomplete adjuvant. The animals were bled from an ear vein prior to administration of booster injections, beginning 4 weeks after primary immunization. Serum was separated from clotted blood by centrifugation and stored in aliquots at −20°C. Antisera were characterized by Ouchterlony double immunodiffusion in 1% agar gels containing 0.5% Triton X-100/150 mM NaCl/10 mM NaP_i (pH 7.4)/0.01% NaN_3 [16].

Immunoprecipitation. For immunoinhibition titration, submitochondrial particles were solubilized by incubating at 3.3 mg protein/ml in 1.67% (w/v) Triton X-100/50 mM NaP_i (pH 7.4) for 10 min at 0°C. In control experiments 90–95% of

total protein and cytochrome oxidase activity remained in the supernatant following ultracentrifugation at the speed employed for isolating submitochondrial particles, indicating effective solubilization of the membranes. Aliquots of the preincubation mixture were subsequently diluted with varying amounts of antiserum and/or 150 mM NaCl containing 1% bovine serum albumin to give final concentrations of 1 mg/ml submitochondrial particles, 0.5% Triton X-100, 15 mM NaP_i (pH 7.4) in a constant final volume (0.1–0.4 ml) in microfuge tubes. Samples were incubated overnight (16–18 h) at 4°C. Immunoprecipitates were collected by centrifugation for 5 min in a Beckman microfuge. Supernatants were stored on ice, and aliquots were used for cytochrome oxidase assays. Prior to determination of protein or electrophoretic analysis, immunoprecipitates were washed twice by resuspension and centrifugation in 0.2 M KP_i/1% Triton X-100 (pH 7.5) and twice again in 150 mM NaCl/10 mM NaP_i (pH 7.5). For protein determination, washed immunoprecipitates were dissolved in 0.1 ml of 1 M NaOH and protein measured by the Lowry procedure.

Electrophoresis. For electrophoretic analysis, washed immunoprecipitates or purified cytochrome oxidase were dissociated in 30 μl of 62.5 mM Tris-HCl, 20% glycerol, 4% SDS, 1% mercaptoethanol (pH 6.8) by incubation at 37°C for 1 h [17]. Electrophoresis was conducted on slab gels (80 × 140 × 2.7 mm), prepared as described by Merle and Kadenbach [17], using a Pharmacia apparatus. The separating gel contained 16% acrylamide/0.5% bisacrylamide/0.375 M Tris-HCl/0.1% SDS/13% glycerol/3.6 M urea at pH 8.8. The stacking gel (1 cm) contained 9.6% acrylamide/0.3% bisacrylamide/0.1 M Tris-HCl/0.1% SDS at pH 6.8. The running buffer was 0.025 M Tris-HCl/0.192 M glycine/0.1% SDS at pH 8.3. Gels were run at 60 V for 30 min, then at 125 V for 17 h, with cooling to maintain 15–18°C. Gels were fixed 4 h in methanol/water/acetic acid (5 : 5 : 1, v/v/v) stained 1 h in 0.2% Coomassie Blue R-250 in 50% methanol/10% acetic acid preheated to 50°C [18], and destained electrophoretically in 25% methanol/10% acetic acid.

Enzyme assays. Cytochrome oxidase activity was assayed by either a polarographic [19] or spectrophotometric [20] procedure in various experiments. The polarographic procedure employed a Clark oxygen electrode in a closed chamber of 1.6 ml volume at 25°C. The medium contained 50 mM KP_i, 1% Tween-80, 0.1 mM EDTA at pH 7.0 with 100 μM cytochrome *c* (Type VI, Sigma) and 20 mM ascorbate. For the spectrophotometric assay, oxidation of 40 μM reduced cytochrome *c* was followed at 550 nm in a similar medium, except that the pH was 5.7 [15]. Stock solutions of reduced cytochrome *c* were prepared using dithionite, followed by extensive dialysis. Succinate dehydrogenase was assayed spectrophotometrically by a standard procedure [4,21].

Data analysis. Least-squares linear regression analysis was used to determine the slope of antibody inhibition curves (plots of % activity versus amount of antiserum). Data points within the range of 100%–25% activity were used in the calculations. I_{50} values, the amounts of antiserum needed to decrease the initial activity by one-half, were determined from such lines. Statistical significance of differences between samples from alcoholic and control rats was evaluated by the paired *t*-test.

Results

Characterization and specificity of the antiserum

Several tests were used to characterize the antiserum obtained from rabbits that had been immunized with purified rat liver cytochrome oxidase. The antiserum inhibited cytochrome oxidase by precipitating the enzyme and thus removing it from solution. This occurred with both purified rat liver cytochrome oxidase preparations and detergent extracts of submitochondrial particles. Preimmune serum did not cause precipitate formation and did not inhibit oxidase activity (Fig. 1). Spectral analyses verified that heme aa_3 was removed from solution on formation of the immunoprecipitates.

Two rabbits were immunized and used in this study; both gave qualitatively similar results. The highest titer antiserum was found 6 weeks following primary immunization. After this time, titers declined despite repeated booster injections. About 60 μl of antiserum was necessary to precipitate 1 nmol of cytochrome oxidase in the best case. Such a value is comparable to those found by other

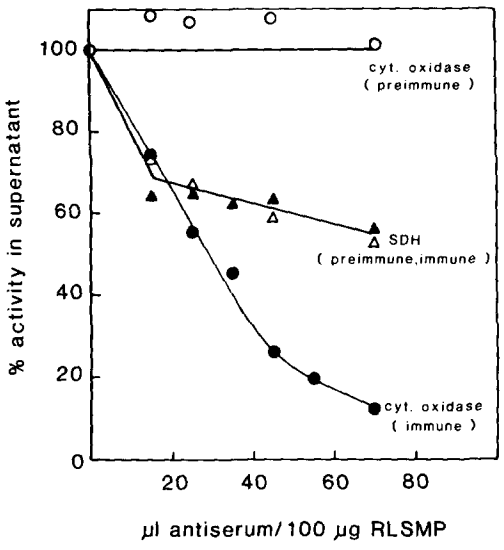

Fig. 1. Specificity of antiserum. Rat liver submitochondrial particles (RLSMP) (0.1 mg protein) were solubilized as described in Experimental procedures and incubated overnight with varying amounts of rabbit serum obtained prior to (open symbols) or after (closed symbols) immunization with purified rat liver cytochrome (cyt.) oxidase. Precipitates formed only in samples containing immune serum. Following centrifugation, activities of cytochrome oxidase (circles) and succinate dehydrogenase (triangles) were measured spectrophotometrically, using aliquots of the supernatants. SDH, succinate dehydrogenase.

investigators for rabbit antisera to bovine heart [22] or rat liver [23] cytochrome oxidase.

Specificity of the antiserum was examined by double immunodiffusion studies. The antiserum formed a single precipitin line when tested against purified cytochrome oxidase or Triton extracts of submitochondrial particles. These formed 'lines of identity' when tested in adjacent wells. Preimmune serum did not form any precipitin bands.

As an additional check of antibody specificity in immunoprecipitation experiments with submitochondrial particles, we measured succinate dehydrogenase activity as an independent marker for the inner membrane. In this case, both preimmune and immune serum caused equivalent partial inhibition of succinate dehydrogenase (Fig. 1). Thus, the inhibition of succinate dehydrogenase appeared to be a non-specific effect of rabbit serum, rather than formation of an antibody-antigen complex. The specific inhibition of cytochrome oxidase by the antiserum, and lack of specific effect on succinate dehydrogenase, verified that the antiserum was not directed against a contaminating membrane structural component (e.g., lipid) common between these proteins.

In order to characterize further the reactivity of the antiserum, immunoprecipitates were examined by electrophoresis and compared to purified oxidase. Our preparation of rat liver cytochrome oxidase exhibited a subunit profile generally similar to that previously reported for this gel system [17,24]. In our preparation, subunit III exhibited weak staining intensity. In addition, subunits VII*a*, *b* and *c* and VIII were not resolved in the usual 16.5% gel, but were partially separated on 18.5% gels [24]. The preparation exhibited a few high molecular weight bands, which probably represented contaminants or aggregates, along with the known subunits of cytochrome oxidase. Immunoprecipitates derived from purified cytochrome oxidase demonstrated the characteristic subunit bands of the native enzyme (Fig. 2), as well as IgG bands. Two additional heavily staining bands, which ran between oxidase subunits I and II, were found in immunoprecipitates. These bands appeared to represent rabbit serum proteins which co-precipitate with the antigen-antibody

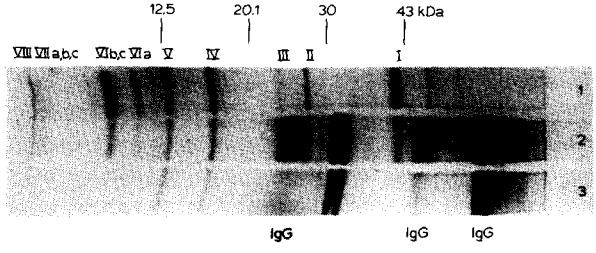

Fig. 2. Electrophoretic analysis of immunoprecipitates obtained with undissociated and dissociated cytochrome oxidase. For immunoprecipitation, purified rat liver cytochrome oxidase (29 µg) was incubated overnight at 4°C with antiserum in buffer containing 0.5% Triton X-100 (undissociated), or preincubated in 4% SDS (1 h/37°C) then diluted with buffer and mixed with antiserum to give final detergent concentrations of 0.1% SDS/0.5% Triton X-100 (dissociated). Immunoprecipitates were analyzed by electrophoresis as described in Experimental procedures. Lane 1: native rat liver cytochrome oxidase; lane 2: immunoprecipitate of undissociated cytochrome oxidase; lane 3: immunoprecipitate of dissociated cytochrome oxidase. Migration positions of molecular-weight standard proteins and IgG are noted on the axis. The narrow band near the bottom of the gel is due to lipid-detergent micelles.

complex, and have been observed in other studies [23]. To determine which particular subunits of the native enzyme were recognized by the antiserum, the enzyme was first dissociated by incubation in SDS and subsequently immunoprecipitated [23]. In this case, only subunits IV and V were found in the precipitate (Fig. 2). A similar pattern of antigenic reactivity has been reported by Merle et al. [23], who found that subunits IV, V, and VI*b* were precipitated from SDS-dissociated oxidase by various rabbit antisera against the holoenzyme.

Immunoinhibition studies with submitochondrial particles

In a previous study, we have established that there is no systematic difference between alcoholic and control rats in the yield or purity of submitochondrial particles obtained by the preparative procedure [2]. Both preparations are equally representative of the composition of the mitochondrial membrane in intact cells, as well as isolated mitochondria [2,3]. Our approach for measuring cytochrome oxidase was that of immunoinhibition titrations [25]. In the absence of antibody submitochondrial particles from alcoholic rats displayed only about one-half the apparent cytochrome oxidase activity per mg membrane protein as those from controls (Fig. 3), in agreement with previous findings [2]. Titrations of a fixed amount of anti-oxidase serum with increasing amounts of submitochondrial particles from alcoholic or control rats showed that similar percentages of added oxidase activity were recovered in supernatants after immunoprecipitation (Fig. 3). After incubation with antiserum, the plots of absolute activity versus amount of membrane protein were displaced toward higher protein concentration by equivalent amounts with both alcoholic and control rats. Both titration curves showed an upward deflection at equivalent amounts of submitochondrial particle protein added to the incubation. This pattern is indicative of a similar degree of immunologic reactivity per mg membrane protein in both preparations.

Titrations of a fixed amount of submitochondrial particles with increasing antiserum likewise showed that similar amounts of antiserum were necessary to inhibit the respective activity of each membrane preparation by 50% of its original value (Fig. 4). Substantial variability between different

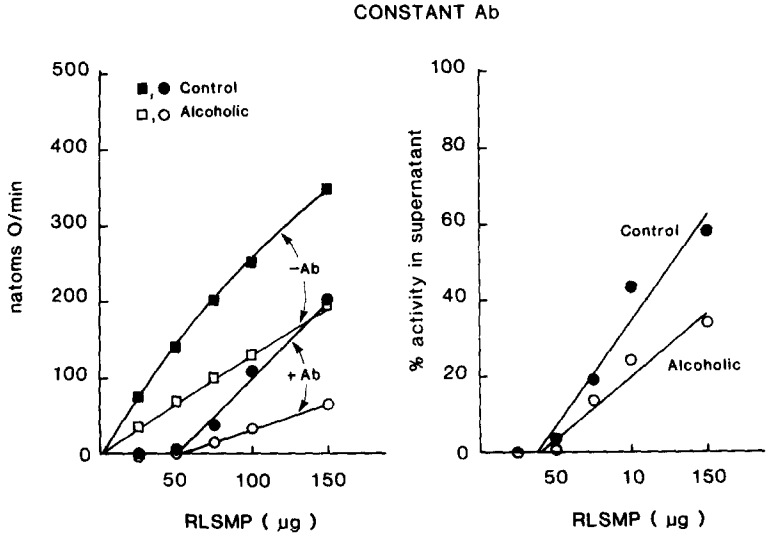

Fig. 3. Extract titration at fixed antibody concentration. Left: rat liver submitochondrial particles (RLSMP, 0–0.6 mg protein) were solubilized as described in Experimental procedures and incubated overnight with 100 µl of antiserum (Ab, antibody or antiserum) in a final volume of 0.4 ml. Following centrifugation, 0.1 ml aliquots of the supernatants were tested for cytochrome oxidase activity by a polarographic assay (circles). Activity in the absence of antiserum (squares) was measured similarly using an equivalent amount of protein for each incubation. Right: results are expressed as the percentage of added activity recovered in the supernatant after immunoprecipitation (i.e., natoms O/min, $+Ab/-Ab \times 100\%$). Control, closed symbols; alcoholic, open symbols.

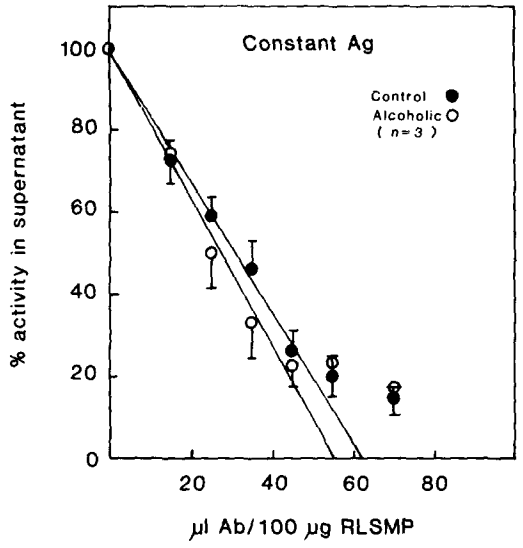

Fig. 4. Antibody titration at fixed extract protein antigen concentration. Rat liver submitochondrial particles (RLSMP) (0.1 mg protein) were solubilized as described in Experimental procedures and incubated overnight in a final volume of 0.1 ml. Following centrifugation, cytochrome oxidase activity remaining in the supernatant was measured by a spectrophotometric assay. Symbols indicate mean and S.D. for replicate experiments done with one particular batch of antiserum and three different pairs of submitochondrial particles from control (closed circles) and alcoholic (open circles) rats. Ag, antigen, Ab, antiserum.

batches of antiserum was observed with respect to the amount needed to cause 50% inhibition of activity (I_{50}), owing to different titers. However, all titration experiments were done as pairs of alcoholic and control preparations with a given batch of antiserum. For nine such titration experiments, the ratio of I_{50} (control)/I_{50} (alcoholic) was 1.10 ± 0.16 (S.D.), indicative of similar relative immunologic reactivity for both types of membranes.

The absolute amounts of protein present in immunoprecipitates were comparable with both alcoholic and control preparations, in either fixed antiserum or fixed membrane protein titration experiments (not shown). This finding corroborated the immunoinhibition titration data and provided additional evidence that both alcoholic and control membranes had quantitatively similar immunologic reactivity.

Electrophoretic analysis of immunoprecipitates

Immunoprecipitates derived from submitochondrial particles showed prominent bands corresponding to cytochrome oxidase subunits, when resolved on electrophoresis gels under dissociating conditions. The characteristic oxidase subunit profile was readily recognizable, especially in the low molecular weight region. However, several other bands, which arose from co-precipitation of other membrane and serum proteins, were also evident. The electrophoretic profiles of immunoprecipitates from alcoholic and control rats were similar with respect to both band locations and staining intensities. Interestingly, immunoprecipitates from alcoholic rats showed one additional band in the region of oxidase subunit IV that was not present in samples from control rats (Fig. 5). On some gels, the band corresponding to oxidase subunit IV appeared more intense in samples from alcoholic rats compared to controls, and was not resolved into a double band. The identity of this extra protein band remains to be established.

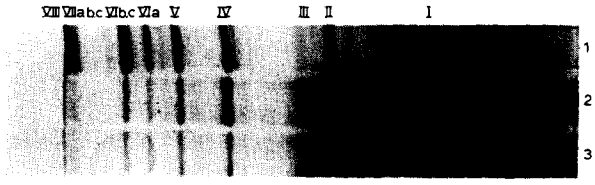

Fig. 5. Electrophoretic analysis of immunoprecipitates from submitochondrial particles. Immunoprecipitates obtained from Triton-solubilized submitochondrial particles were analyzed by electrophoresis as described in Experimental procedures. Lane 1: native rat liver cytochrome oxidase; lane 2: immunoprecipitate from an alcoholic rat; lane 3: immunoprecipitate from the pair-fed control rat.

Discussion

Previous studies, confirmed in the present experiments, have indicated that the amount of enzymatically functional cytochrome oxidase in mitochondrial membranes from ethanol-fed animals is only about half that of pair-fed controls, using total membrane protein as the reference. Employing a specific antiserum against rat liver cytochrome oxidase as a probe for the protein portion of this hemoprotein, we have now shown that cytochrome oxidase content, expressed as immunologic reactivity per mg membrane protein, is comparable in submitochondrial particles

from alcoholic and control rats. The evidence for this conclusion derives from (1) the similarity between alcoholic and control preparations in immunoinhibition titrations, using either fixed antiserum or fixed membrane protein concentrations; (2) the equivalent amounts of protein recovered in immunoprecipitates; and (3) the comparable electrophoretic profiles of the immunoprecipitates. Thus, the protein moiety of cytochrome oxidase recognizable by the antiserum must be present in similar amounts within mitochondrial membranes of alcoholic and control rats. Together, the biochemical and immunochemical data suggest the presence of an inactive form of cytochrome oxidase in mitochondrial membranes of rats fed ethanol. That is, cytochrome oxidase biochemical reactivity is one-half but cytochrome oxidase immunologic reactivity is unchanged after alcohol treatment. Membranes from alcoholic animals apparently contain a mixture of approx. half fully active and half fully inactive forms of cytochrome oxidase. The inactive form of oxidase seems to be devoid of heme, since the latter is undetectable spectrophotometrically either in native membrane preparations [2] or after extraction as the pyridine hemochromogen [26]. The inactive form of the enzyme may be associated with a subtle modification in subunit structure, perhaps subunit IV, as suggested by the electrophoretic analysis of immunoprecipitates. No specific function for subunit IV has yet been established [27]. Subunits I and II have been identified as the major heme binding subunits of cytochrome oxidase [27,28], though some studies have also suggested a role for subunit IV in this process [27,29].

The molecular mechanism underlying formation of the suggested inactive form of cytochrome oxidase in ethanol-fed animals remains to be elucidated. Ethanol consumption may interfere with the assembly of cytochrome oxidase, perhaps by affecting heme attachment. Alternatively, it might cause an inactivation of pre-existing functional cytochrome oxidase. Previous studies have demonstrated that chronic ethanol consumption decreases the ability of mitochondria to incorporate amino acids into membrane proteins [8,30,31], some of which have been suggested to be subunits of cytochrome oxidase [31]. The present findings do not rule out ethanol-related effects on mitochondrial protein synthesis as being involved in the alteration of cytochrome oxidase, but suggest that subunits of the oxidase are present in normal amounts in hepatic mitochondria of alcoholic rats. It is possible, however, that the oxidase subunits may be assembled incorrectly in alcoholic animals.

Although considerable progress has been made toward delineating the sequence of molecular events involved in the assembly of cytochrome oxidase and other mitochondrial proteins, many aspects of this process remain unknown [32–34]. In particular, relationships between insertion of the protein into the lipid bilayer and heme attachment have not been clarified. Thus, alterations in membrane lipids associated with ethanol consumption may play a role in modulating the assembly of functional cytochrome oxidase. The existence of a heme-deficient form of oxidase in submitochondrial particles from alcoholic animals does suggest that during normal biogenesis oxidase subunits may be fully assembled and inserted into the membrane prior to heme attachment.

The present results may shed light on one particularly puzzling set of observations. Because by functional criteria several major mitochondrial membrane proteins appear decreased by 30–50% after chronic ethanol consumption [1–4], and other proteins do not appear compensatorily increased, one would anticipate a substantial decrease in total mitochondrial protein per gram liver. By contrast, the amount of total mitochondrial protein per gram liver has been reported to be unchanged after chronic ethanol consumption [35]. However, if other membrane proteins are also present in inactive forms, analogous to the condition of cytochrome oxidase, then both sets of data would be readily understood. Thus, results of the present immunochemical study complement previous biochemical studies and provide new insight into the actions of ethanol at the molecular level.

Acknowledgements

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