Human Sulfite Oxidase Deficiency

CHARACTERIZATION OF THE MOLECULAR DEFECT IN A MULTICOMPONENT SYSTEM

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ABSTRACT Frozen liver tissue from an individual identified several years ago as sulfite oxidase deficient has been reexamined in light of new knowledge which has been obtained regarding the enzyme. It has been established that hepatic molybdenum levels and xanthine oxidase activity were within normal values and comparable to those observed in control samples preserved from the original study along with the deficient tissue sample. The ability of the patient's liver to synthesize the specific molybdenum cofactor required for activation of de-molybdo sulfite oxidase also appears to have been unimpaired. Using an antibody preparation directed against rat liver sulfite oxidase which also inhibits and precipitates the human enzyme, it has been determined that cross-reacting material with determinants recognized by inhibiting antibodies is absent in the liver sample from the deficient patient. Immunodiffusion experiments gave strong precipitin bands against the control liver extracts, but showed no detectable precipitin reaction between the deficient liver extract and the antibody preparation. The relationship of these findings to a second patient recently identified as sulfite oxidase deficient and to an animal model of the disease are discussed.

INTRODUCTION

Several years ago a new deficiency disease was described by Irreverre et al., Mudd et al., and Perry et al. (1–3). The enzymatic defect identified was that of sulfite oxidase which catalyzes the terminal reaction in the pathway of oxidative degradation of sulfur amino acids. The patient studied was a 2½ yr old boy with severe brain damage, mental retardation, and dislocated lenses. Urinary excretion of abnormally high levels of S-sulfo-L-cysteine, sulfite and thiosulfate, and very little inorganic sulfate was suggestive of an impaired ability to oxidize sulfite to sulfate. Liver, kidney, and brain tissue were assayed postmortem for sulfite oxidase activity, and none was detected.

At the time these studies were undertaken by Mudd et al. (2) very little information was available regarding the nature of the enzyme sulfite oxidase. Since then, the enzyme has been purified from several sources and studied in much detail.

Sulfite oxidase is a molybdohemoprotein with a mol wt of 110,000–120,000 daltons (4–6). The molybdenum in the rat liver enzyme has been studied by electron paramagnetic resonance spectroscopy and been shown to exhibit a characteristic pH and anion-sensitive spectrum, both in the purified state when reduced with sulfite and in the crude homogenate stage (7). Sulfite oxidase is a soluble mitochondrial enzyme located in the intermembrane space and transfers electrons from sulfite into the electron transport chain via cytochrome *c* (8–10).

Studies in this laboratory have shown that it is possible to create an experimental model of sulfite oxidase deficiency in the rat (11–13). Administration of high levels of tungsten antagonizes molybdenum utilization in this species and leads to a simultaneous deficiency of sulfite oxidase and a second molybdoenzyme, xanthine oxidase. The activities of both enzymes in several tissues undergo dose- and time-dependent decreases, yet synthesis of the de-molybdo apomolecules proceeds normally. Incorporation of tungsten into the molybdenum site of a portion of the sulfite oxidase molecules has been observed, but the tungsten derivatives, like the apoenzyme molecules are inactive.

Received for publication 24 February 1976 and in revised form 30 April 1976.

Recently we have made use of the de-molybdo sulfite oxidase molecules to study the processes involved in reconstitution of the molybdenum center.[1] With the reappearance of sulfite oxidase activity as a simple assay of proper reconstitution, we have shown that before molybdate can be incorporated into the sulfite oxidase molecule, it must be modified or activated to the level of a molybdenum cofactor. The cofactor appears to be synthesized in some excess in control animals and accumulates in a stable form within the liver cell. From these studies we are able to define several distinct steps in molybdenum utilization in animals, all of which must be operating properly for sulfite oxidase activity to be expressed: molybdenum in the diet must be efficiently absorbed (presumably as molybdate) by the organism, transported to the proper tissues, and activated to the level of a cofactor for incorporation into the apoprotein itself. It is quite likely that these several processes are under the control of independent genetic loci and that a defect at any of these several points or in the sulfite oxidase protein itself could result in a phenotype characterized as sulfite oxidase deficient.

With these new observations in hand, a reinvestigation of the defect identified by Mudd et al. (2) as sulfite oxidase deficiency was considered highly important. Such a study was made possible as the result of careful preservation of autopsy tissues taken from the deficient individual as well as critical tissues from patients who served as controls in the original studies. Biochemical and immunochemical analysis of these samples has allowed characterization of the specific lesion responsible for sulfite oxidase deficiency in this individual as described below.

METHODS

The purification and characterization of sulfite oxidase from human liver are described in the preceding article (14). Studies reported in detail there have established that the human enzyme cross-reacts very strongly with antibody directed against rat liver sulfite oxidase.

The spectrophotometric assay of sulfite oxidase activity with cytochrome *c* as electron acceptor is described in the accompanying article (14). Xanthine oxidase activity was assayed by spectrophotometric measurement of uric acid production at 295 nm. Cuvettes contained 200 μl extract, 0.75 mM xanthine, and 0.5 mM NAD^+ in 1 ml of 0.05 M potassium phosphate buffer, pH 7.8. Conversion of xanthine dehydrogenase in human liver into an oxidase during tissue storage and preparation of the extract (15) does not affect the rate of uric acid formation when both NAD^+ and oxygen are available as electron acceptors. 1 U of xanthine oxidase activity is defined as the amount of enzyme which produces an absorbance change of 1.0/min under the above conditions. By this definition 10 U correspond to 1 μmol of uric acid produced per minute. All enzyme assays were done on a Cary 14 spectrophotometer (Cary Instruments, Fairfield, N. J.) at room temperature. Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as standard.

Molybdenum analysis was done on tissue samples ashed overnight at 450°C in a muffle furnace. Determinations were made by a colorimetric procedure described previously (12) and by atomic absorption spectroscopy using a Perkin Elmer model 107 spectrometer fitted with a heated graphite atomizer (HGA model 2000). Perkin Elmer Corp., Norwalk, Conn.

Techniques for preparation of antibody and development of immunodiffusion plates have been described (14).

Tissue samples from the sulfite oxidase deficient patient (T. A.) and from two patients who served as controls in the earlier studies (G. W. and P. M.) were kindly provided by Dr. S. Harvey Mudd of the National Institute of Mental Health. Tissues were stored at −50°C at all times until used for the studies described. A normal human liver obtained at autopsy and frozen for less than 2 mo (C-1) was used for all preliminary experiments to conserve the more valuable tissue samples and also served to identify artifacts which may have arisen during prolonged storage of these tissues.

RESULTS

Sulfite oxidase activity in normal human liver ranges from 10 to 25 U/g wet weight of the tissue. For assay of this activity in the frozen liver samples, 0.2 g of tissue was homogenized in 1.0 ml of 0.01 M potassium phosphate buffer, pH 7.8 using a ground-glass tissue homogenizer. The homogenate was centrifuged at 35,000 *g* for 20 min, and the supernatant fraction was filtered through Sephadex G-25 (Pharmacia Fine Chemical, Inc., Piscataway, N. J.) to remove endogenous reductants which produce high rates of sulfite-independent reduction of cytochrome *c*. Assay of this filtered supernate indicated that sulfite oxidase activity in the frozen control samples was within the normal range and thus had not deteriorated upon prolonged storage (Table I). Activity in liver from patient T. A. was below the limits of detection of the assay, or less than 0.1 U/g. Thus,

TABLE I

Levels of Molybdenum and Molybdoenzymes in Control and Sulfite Oxidase Deficient Livers

	C-1*	G. W.‡	P. M.‡	T. A.§
Sulfite oxidase, *U/g*	13.5	27.5	13.5	0‖
Xanthine oxidase, *U/g*	0.76	0.33	0.46	0.51
Molybdenum-atomic absorption, *μg/g*	0.92	0.89	0.38	0.33
Molybdenum-colorimetric, *μg/g*	1.01	0.84	0.33	0.31

* Control adult, male; tissue obtained at time of present study.
‡ Controls studied by Irreverre et al., Mudd et al., and Percy et al. (1–3): G. W., adult, female; P. M., 4 mo of age, male.
§ Sulfite oxidase deficient patient, 2½ yr old, male.
‖ No activity detected.

[1] J. L. Johnson and K. V. Rajagopalan. Manuscript in preparation.

the enzymatic defect identified by Mudd and co-workers (2) is confirmed by our assay procedure. Xanthine oxidase activity, assayed in the same supernatant preparations, was quite similar in all three control samples. The activity of this enzyme in liver deteriorates quite rapidly if autopsy is delayed or liver specimens are not frozen immediately.[3] The consistent values which were observed indicate that the activity is not adversely affected by long storage at − 50°C and furthermore suggest that the liver samples were frozen promptly and in good condition. Hepatic xanthine oxidase activity in T. A. is well within the normal range indicating that the patient was not suffering from a generalized molybdenum deficiency. This was confirmed by direct molybdenum analysis both by atomic absorption and a colorimetric procedure (Table I). When evaluating the data on hepatic molybdenum levels, it becomes important to consider the age of the individual at the time of death. Schroeder et al. (17) have studied hepatic molybdenum levels in humans and reported that the metal is present in relatively low amounts in the newborn, rises to a peak corresponding to about 1.1 μg/g in the second and third decades of life and shows a moderate decrease thereafter. Our results are entirely consistent with these observations. The higher values obtained from individuals C-1 and G. W. are as expected in adult tissue. P. M. and T. A. died at 4 mo and 2½ yr of age, respectively, and had less than adult levels of hepatic molybdneum. Patient T. A. thus displayed apparently normal molybdenum accumulation for his age.

Normal xanthine oxidase and molybdenum values in the liver of the sulfite oxidase deficient patient might suggest that overall molybdenum metabolism in this individual was not defective. Xanthine oxidase, like sulfite oxidase, requires an activated special form of molybdenum;[1] in fact it has been postulated by Nason et al. (18) that molybdoenzymes in general share a common cofactor. Certainly if this is the case, normal xanthine oxidase levels in patient T. A. would indicate that a defect in the system required to activate molybdate is not the lesion responsible for sulfite oxidase deficiency. For confirmation, a direct test for the presence of sulfite oxidase cofactor in the sulfite oxidase deficient liver sample was undertaken. Apoenzyme was isolated from livers of tungsten-treated rats (12) by purification through the DEAE-cellulose column stage and served as the reconstitutable material for assay of the sulfite oxidase molybdenum cofactor. Small samples of control rat liver and of the various human liver specimens were homogenized in 5 vol of 0.01 M potassium phosphate buffer, pH 7.8. 25-μl microliter samples of each homogenate (representing 4.2 mg liver) were combined with 2 μg of apo sulfite oxidase in a total volume of 200 μl. Previous studies with the rat liver system have shown that incubation of control rat liver homogenate with apo sulfite oxidase at 37°C leads to a time-dependent reappearance of sulfite oxidase activity which reaches completion within 40 min.[1] Reconstitution at completion is linear with respect to cofactor added until apoenzyme in the reaction mixture is saturated. 2 μg of apo sulfite oxidase can be reconstituted to yield a maximum of 1.2 U of activity or 6 U/ml in the incubation mixture. As can be seen in Table II studies with C-1 established that human liver, like rat liver, does accumulate a molybdenum cofactor capable of activating apo sulfite oxidase in the in vitro system. Moreover, liver tissue from control G. W. contained a comparable level of endogenous cofactor capable of sulfite oxidase reconstitution. Liver samples from P. M. and T. A. showed somewhat less reconstituting ability, but this is not surprising in view of their lower liver molybdenum levels. The crucial conclusion which can be drawn is that patient T. A. had enough cofactor per gram of liver tissue to activate considerably more sulfite oxidase than is normally present in human liver.

TABLE II

Levels of Endogenous Molybdenum Cofactor in Control and Sulfite Oxidase Deficient Livers

	Rat	C-1	G. W.	P. M.	T. A.
U/ml incubation mixture	2.22	1.51	1.58	1.15	0.89
U/g liver	106	72	75	55	42

Liver homogenates were incubated with apo sulfite oxidase at 37°C for 45 min. Rates are corrected for activities of homogenates alone and of apoenzyme alone.

It appears then that the defect responsible for sulfite oxidase deficiency in T. A. must in some way relate to the structural protein of the enzyme. Either the protein is not synthesized, or is synthesized in extremely low amounts or in an altered form. As a sensitive test for inactive cross-reacting material in the liver of patient T. A., an antibody protection experiment was undertaken using rabbit antiserum directed against rat liver sulfite oxidase. It has been established (14) that this antibody preparation contains populations of antibody molecules which will recognize human liver sulfite oxidase as evidenced by inhibition of sulfite oxidase activity with cytochrome *c* as electron acceptor and precipitation of the sulfite oxidase molecules. Any inactive protein molecules related to or derived from sulfite oxidase in an extract of the deficient liver could also be recognized by the antibody population. Thus, when a control extract and the sulfite oxidase deficient extract are incubated to-

[3] S. Adler and K. V. Rajagopalan. Unpublished observation.

TABLE III

Assay for Cross-Reacting Material in Sulfite Oxidase Deficient Liver

Incubation mixture	Units added	Units remaining	Inhibition
			%
Experiment 1:			
Control homogenate	0.0375	0.0356	5
Control homogenate +antibody	0.0375	0.0060	83.1
Control homogenate +deficient homogenate +antibody	0.0375	0.0064	82.1
Experiment 2:			
Control homogenate	0.0794	0.0789	1
Control homogenate +[antibody]	0.0794	0.0161	79.7
Control homogenate +[deficient homogenate +antibody]	0.0794	0.0086	89.2

Reaction mixtures were incubated at 4°C for 40 min, centrifuged for 10 min and assayed for sulfite oxidase activity. Reactants in brackets were preincubated for 40 min and centrifuged; supernatant fractions were combined with control homogenate, incubated, centrifuged, and assayed.

gether with antiserum, the presence of cross-reacting material in the liver sample from T. A. would be detected as a lessened inhibition by antibody. As shown in Table III (experiment 1), no significant protection was noted.

In a second experiment, the deficient extract was preincubated with the antiserum, centrifuged, and the supernatant fraction added to the control homogenate. In this case, the possible problem of inactive material present in antibody complex being displaced by active sulfite oxidase was eliminated since the antigen-antibody complexes, if formed, would have been removed by centrifugation. Also with such a design, the degree of protection expected would be greater than in experiment 1 since inactive cross-reacting material has free access to antibody during the initial incubation and does not compete directly with active enzyme molecules. As shown in Table III (experiment 2), preincubation of antibody with deficient liver did not at all protect the control enzyme from antibody inhibition.

Thus the antibody protection experiments clearly demonstrate that there is no significant amount of cross-reacting material in the sulfite oxidase deficient liver that retains the critical determinant(s) recognized by the inhibiting antibody population. Although antibody protection is a sensitive test for cross-reacting material, it has the severe limitation that cross-reacting material without these determinants would be undetected. A more general test for cross-reacting material would be one where a minimum number of determinants must be retained. Since retention of 3 or 4 of the 10–20 determinants expected on the native molecule would allow formation of a precipitating antigen-antibody complex, several attempts were made to detect such a precipitate when antiserum was added to an extract of the deficient liver. Fig. 1 shows an immunodiffusion plate where extracts of control and deficient liver were allowed to react with the antibody preparation. While the control extract gives a strong precipitin band with the antirat liver sulfite oxidase antiserum, no trace of cross-reaction is seen with the deficient liver extract. An important aspect of this experiment is that the extracts applied were in as crude a stage as could be adapted to the immunodiffusion technique. Small samples of liver were homogenized in hypotonic buffer, centrifuged at 100,000 *g* for 1 h and concentrated by lyophilization. By this approach we have carefully avoided any fractionation steps which might result in loss of cross-reacting material.

A potentially more sensitive technique for monitoring antigen-antibody complex formation in a crude extract of the deficient liver is a complement fixation assay. This has been explored in some detail and does not appear to be applicable to this situation. The crude mixture of proteins in the extract used as the source of antigen is extremely anticomplementary; that is, complement consumption is high with antigen alone. The amount of complement fixed by the antigen-antibody complex is less than that destroyed by antigen alone making quantitation of cross-reacting material by this method very difficult.

Processes of heme metabolism as they relate to sulfite oxidase have not yet been investigated in detail. However, correct synthesis and incorporation of the cytochrome b_5 prosthetic group must be considered essential for expression of sulfite oxidase activity. Thus,

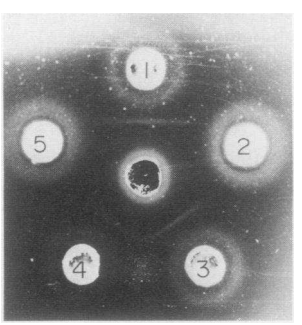

FIGURE 1 Ouchterlony immunodiffusion of extracts of liver tissue from control and sulfite oxidase deficient patients. Extracts were prepared as described in the text. Wells 1 and 3 each contained 20 μl of liver extract from control G. W.; wells 2 and 5 each contained 20 μl of liver extract from the sulfite oxidase deficient patient T. A.; well 4 contained 20 μl of liver extract from control P. M. The center well contained 20 μl of antiserum directed against purified rat liver sulfite oxidase. The plate was photographed directly without staining.

it is conceivable that patients with a lesion in heme metabolism may also be identified as sulfite oxidase deficient. In patient T. A. no evidence for molybdenum-containing, heme-free protein has been adduced;[3] and general heme metabolism in this individual was not obviously defective. If other instances of sulfite oxidase deficiency are identified, and inactive cross-reacting material is detected, a thorough investigation of molybdenum and heme biosynthesis and utilization would be critical to a delineation of the specific metabolic error.

DISCUSSION

At this point, we have obtained considerable information regarding the metabolic defect expressed as sulfite oxidase deficiency in patient T. A. By a number of criteria, molybdenum metabolism in this indivdual appeared to be normal. The problem was traced rather to the structural protein of the enzyme. The reason for the virtual absence of cross-reacting material has not yet been elucidated. The indications are that the structural protein may not be synthesized at all, but we have not yet ruled out the presence of an altered gene product, in particular one with little or no reactivity with the antibody preparation. In addition, it is possible that prolonged storage of tissue from patient T. A. could have damaged the sulfite oxidase protein and led to a loss of antigenic cross-reactivity. Native enzyme appeared totally stable to the conditions to which it was subjected in the control samples, but somewhat elevated levels of sulfite, as may have been present in the sulfite oxidase deficient liver, could have been detrimental to an incomplete or less stable gene product.

The conclusions reached from this study may or may not be applicable to other instances of sulfite oxidase deficiency as they may be identified. A second patient currently being studied has recently been characterized as sulfite oxidase deficient.[4] Since it is very likely that more than one gene is involved in the expression of sulfite oxidase activity, there is no reason to expect that this individual will show a lesion identical to that in T. A. However, the information gained and the methodology developed in studying frozen tissue samples from the first deficient individual should enable rapid delineation of the specific biochemical defect expressed as sulfite oxidase deficiency in this deficient patient, and others which may be discovered, using small tissue samples obtainable by biopsy procedures.

In an earlier publication from this laboratory (7), it was proposed that the characteristic electron paramagnetic resonance signal of the molybdenum center of sulfite oxidase could be monitored, in addition to enzymatic activity, in biopsy samples. Now with additional information regarding the state of the enzyme in human tissue (14), it is clear that such an approach would not be feasible. Identifying sulfite oxidase molybdenum in crude rat liver preparations is not difficult but approaches the limits of sensitivity of available instrumentation. The much lower levels of sulfite oxidase in human liver, about 5% of that present in rat liver, could not be reliably detected by electron paramagnetic resonance in crude homogenates. Electron paramagnetic resonance studies of more purified preparations could of course be easily undertaken and should provide further interesting information on the molybdenum center of human sulfite oxidase.

A final point to be considered concerns the relation of the experimental animal model of sulfite oxidase deficiency to the human disease as expressed in T. A. and the patient currently under investigation. As is evident from the studies in this report, the animal model is not biochemically rigorous. In the animal system the structural protein of sulfite oxidase is normally expressed but is rendered nonfunctional through the intentional competitive elimination of the molybdenum cofactor. In patient T. A., normal levels of cofactor were present, and the defect was apparently in the structural protein of the enzyme. Nevertheless, the phenotype is the same: tissues from tungsten-treated rats and those from patient T. A. are deficient in sulfite oxidase activity. It is therefore important to evaluate the relevance and applicability of the animal model to studies of the human disease.

The clinical manifestations of sulfite oxidase deficiency in the two patients observed have included neurological abnormalities, mental retardation, and dislocation of the ocular lenses (1).[4] While establishment of definitive symptoms associated specifically with the disease should await studies of additional cases, it is clear that absence of sulfite oxidase activity can be quite detrimental to the well-being of those individuals. As described in earlier publications (11, 19) creation of sulfite oxidase deficiency in rats has been achieved by administration of 100 ppm tungsten in the drinking water. Rats depleted of tissue sulfite oxidase were found to be significantly more susceptible to the toxic effects of injected bisulfite or respired SO_2 (19); but, in the absence of such stress, they were indistinguishable from control animals. A similar lack of effect of sulfite oxidase deficiency was noted in second generation molybdenum-deficient rats

[3] It has not yet been established that heme-free sulfite oxidase from human or rat liver would be precipitated by the antibody preparation. However, in view of the large size of the sulfite oxidase protein (14), we would anticipate that a sufficient number of determinants would be retained in recognizable conformation on the molecule in the absence of the heme prosthetic group.

[4] Dr. Vivian Shih, Massachusetts General Hospital, Boston, Mass. Personal communication.

when pregnant females were administered 100 ppm tungsten (20). In spite of the induced sulfite oxidase deficiency, the second generation tungsten-treated rats were completely normal in number, growth patterns, and survival rates. In the experimental sulfite oxidase deficiency described above, animals retained 2–5% residual activity. While this is decidedly severe when contrasted to the normal levels of the enzyme in rat liver (200 U/g), it is much less marked when compared to the sulfite oxidase content of normal human liver. The observed well-being of rats which retain a partial, but very low, expression of sulfite oxidase activity suggests that while a total absence of sulfite oxidase in humans is apparently detrimental, a partial deficiency may result in less severe symptoms or none at all. Recently we have observed that administration of 400 ppm tungsten in the diet to adult rats for several months results in hepatic sulfite oxidase contents of less than 1% of normal without impairing the health of the animals. However, when pregnant rats were maintained on this level of tungsten, the litters derived from them showed symptoms of toxicity, including poor weight gain and poor survival rates. This finding raises the possibility that exposure of rats to 400 ppm tungsten will give rise to progeny which are much better analogs of human sulfite oxidase deficiency than those described earlier. If toxicity in these second generation tungsten-treated rats can be demonstrated to be specifically linked to sulfite oxidase deficiency, they will provide a rare experimental animal model of a human deficiency disease, and should be extremely valuable in examining the basis of the symptomatology of the disease. This possibility is currently under investigation.

ACKNOWLEDGMENTS

This work was supported by Research grant GM 00091 from the National Institutes of Health (to Dr. Rajagopalan) and by National Institutes of Health Postdoctoral Fellowship ES 00036 (to Dr. Johnson). The authors thank Dr. S. Harvey Mudd for valuable discussion and critical reading of the manuscript.

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