The Structure of a Molybdopterin Precursor

CHARACTERIZATION OF A STABLE, OXIDIZED DERIVATIVE

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An oxidized pterin species, termed compound Z, has been isolated from molybdenum cofactor-deficient mutants of *Escherichia coli* and shown to be the direct product of oxidation of a molybdopterin precursor which accumulates in these mutants. The complete structural characterization of compound Z has been accomplished. A carboxyl function at C-1' of the 6-alkyl side chain can be reacted with 2,4-dinitrophenylhydrazine to yield a phenylhydrazone and can be reduced with borohydride, producing a mixture of two enantiomers, each with a hydroxyl group on C-1'. Compound Z contains one phosphate/pterin and no sulfur. The phosphate group is insensitive to alkaline phosphatase and to a number of phosphodiesterases but is quantitatively released as inorganic phosphate by mild acid hydrolysis. From \(^{31}\)P and \(^{1}H\) NMR of compound Z it was inferred that the phosphate is bound to C-2' and C-4' of a 4-carbon side chain, forming a 6-membered cyclic structure. Mass spectral analysis showed an MH\(^{+}\) ion with an exact mass of 344.0401 corresponding to the molecular formula C\(_{19}\)H\(_{11}\)N\(_{2}\)O\(_{3}\)P, confirming the proposed structure.

All molybdenum-containing enzymes except nitrogenase complex the metal through a unique pterin termed molybdopterin (1). Although direct structural studies of the molybdenum cofactor have been precluded by its extreme lability when released from the molybdoenzymes, complete structural characterization was achieved through the analysis of four inactive derivatives. Two of these derivatives, Form A and Form B, are the products of *in vitro* oxidation of the active cofactor (2). Urothione, the third derivative, is a metabolic product of molybdopterin excreted in urine (3). The fourth was obtained by carboxamidomethyl derivatization of the cofactor (4). The structures of the molybdenum cofactor and molybdopterin, the organic portion of the cofactor, are shown in Fig. 1, as are those of the four derivatives from which the molybdopterin structure was deduced.

The lability of the cofactor may be attributed to the reactivity of the vicinal dithiol function on the side chain of the pterin. The formation of a stable derivative retaining both sulfurs has been successfully accomplished in *vitro* only by gentle anaerobic alkylation of the sulfurs with iodoacetamide (4). The presence of this highly reactive group on the cofactor raises the significant question of how such an unstable molecule is synthesized *in vivo*. Conditions necessary for the release of an intact molybdopterin-molybdenum complex from a molybdoenzyme have been described (5). The metal ligation to the pterin in the complex proved to be transient, however, demonstrating that molybdenum chelation alone could not serve to stabilize the edenthiol during biosynthesis. Other possibilities for stabilization are that molybdopterin intermediates are protein-bound or that the edenthiol is generated in the last step of molybdopterin biosynthesis directly preceding molybdenum insertion and incorporation of the resulting molybdenum cofactor into apoenzymes.

Structural analysis of molybdopterin precursors would be expected to yield crucial information about the cofactor biosynthetic pathway. A number of molybdopterin mutants have been identified which accumulate a molybdopterin precursor. The *Escherichia coli* mutants *chlM* and *chlN* and the *Neurospora cressa* mutant nit-1 all produce a low molecular weight species which can be converted to molybdopterin when incubated with the high molecular weight fraction of the *E. coli* molybdopterin mutant *chlA* (6, 7). The precursor produced by these mutants was partially characterized but was difficult to purify because of its lability under aerobic conditions, and direct structural analysis was not accomplished. However, indirect evidence suggested that the precursor was converted to an inactive fluorescent 6-alkyl pterin, termed compound Z, when extracts containing the precursor were treated with iodine. First, compound Z could be isolated only from those cofactor-deficient strains which exhibited precursor activity (6). Second, preincubation of an extract containing precursor activity with a *chlA* extract greatly decreased the amount of compound Z produced by subsequent iodine oxidation (8).

The objective of the present studies was to investigate in detail the structure of compound Z and to clarify the nature of its relationship to active molybdopterin precursor. In this paper we present evidence that compound Z is formed directly from the precursor by oxidation in air or with iodine. Definitive structural characterization of compound Z was accomplished by a number of spectral and chemical methods. The data presented in this article are consistent with the structure for compound Z shown in Fig. 2. Even as the study of inactive stable derivatives of molybdopterin proved to be a sine qua non for determining the structure of the cofactor itself, the structure of compound Z is seen to contain information about the chemical nature of the molybdopterin precursor and the reaction that converts it to molybdopterin.

MATERIALS AND METHODS

Chicken intestine alkaline phosphatase was from Worthington. Perchloric acid was from MCB and dithiothreitol from Becham. Dimethyl-d\(_6\) sulfoxide was from Aldrich and NADPH was from Phar-
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MOLYBDENUM COFACTOR

MOLYBDOPTEIN

UROTHIONE

CARBOXYAMIDO MOLYBDOPTEIN

Fig. 1. Structures of the molybdenum cofactor, molybdopterin, and the four inactive derivatives of molybdopterin.

Proposed structure of compound Z.

COMPOUND Z

Aerobic cultures were shaken vigorously during growth, and cells were harvested at early stationary phase. Anaerobic cultures were grown without shaking. Cells were harvested by centrifugation at 5,000 rpm for 25 min in a Sorvall RC-3B centrifuge.

Purification of Compound Z—The pellet from 3 liters of aerobically grown chlM or chlN cells was resuspended in HzO to yield a total volume of 72 ml. The suspension was acidified with 2.4 ml of 4 N HC1, and 7.2 ml of 1% IO, 2% KI in HzO was added. The preparation was maintained at room temperature in the dark for 30 min and then centrifuged at 23,000 x g for 15 min. The supernatant was adjusted to pH 9 with 6 ml of 1.0 N NH4OH and applied to a 2.6 x 8.5-cm column of QAE-Sephadex (acetate form) equilibrated with H2O. The column was washed sequentially with 100 ml of H2O and 250 ml of 0.01 N acetic acid, and compound Z was eluted with 0.01 N HCl. Fractions were monitored for fluorescence on an Aminco-Bowman SPF spectrofluorometer with the excitation wavelength set at 360 nm and emission at 440 nm. Fluorescent fractions were pooled and applied to a Florisil column (2.6 x 8.5 cm) equilibrated with 0.01 N HCl. The column was washed with 0.01 N HCl, and compound Z was eluted with 22.5% acetone in H2O. Fractions were monitored for fluorescence as before, and those containing compound Z were pooled and rotoevaporated to dryness. Final purification steps employed HPLC. The sample was chromatographed successively in three solvents: 5% methanol, pH 5; 1 mM ammonium acetate, pH 5; and H2O acidified to pH 2 with trifluoroacetic acid.

Preparation of nit-1 Extracts—N. crassa nit-1 (allele 34547) spores were stored in desiccated silica at -70 °C. Cells were grown, induced for nitrate reductase, and harvested as described previously (9). Cell-free extracts were prepared with a Duall glass grinder (7). Sephadex G-25 excluded and included fractions were obtained using a Pharmacia PD-10 column equilibrated and developed with 0.10 M potassium phosphate buffer, pH 7.4, containing 5 mM EDTA and 1 mM dithiothreitol.

Molybdopterin Precursor Assay—The chlAl extracts used in the precursor assays were prepared by suspending the cell pellet in 10 mM potassium phosphate, pH 7.4, (5 ml/g cells) and passing the suspension through a French pressure cell. The broken cell suspension was then centrifuged at 17,000 x g for 15 min. Aliquots of 1.0 ml of the supernatant were stored frozen at -20 °C. The frozen samples were thawed and diluted 1:1 with the same phosphate buffer before use.

Reconstitution mixtures consisting of 40 µl of an appropriate dilution of the precursor source, 25 µl of unfractionated chlAl extract, 25 µl of nit-1 excluded fraction, and 10 µl of 0.5 M Na2MoO4 were incubated at room temperature for 10 min. Reconstituted nitrate reductase was assayed by the addition of 0.4 ml of a solution of substrates to yield in the 0.5-ml assay mix: 0.2 mM NADPH, 20 mM NaNO3, 10 µM FAD, 5 mM Na2SO3, and 40 mM potassium phosphate, pH 7.4 (10). After incubation at room temperature for 15 min, nitrite was quantitated by a colorimetric assay (9).

HPLC—All HPLC analyses were performed at room temperature with an Alltech C-18 reverse phase column (10 µm, 4.6 x 250 mm) and a Hewlett-Packard 1090 solvent delivery system. Material eluting from the column was monitored for absorbance using a Hewlett-Packard diode array detector and a Hewlett-Packard 3390A integrator.
Packard 1040A diode array detector and for fluorescence with a Hewlett-Packard 1046A programmable fluorescence detector. When isolating active precursor, solvents were degassed by continuous sparging with ultrahigh purity grade helium (Alphagaz). Absorption spectra were recorded on a Shimadzu UV-265 spectrophotometer.

**Phosphorylation Reaction**—Reaction of compound Z with dinitrophenylhydrazine in aqueous solution was carried out at room temperature by addition of 0.2 mM compound in H₂O and 0.2 M 2,4-dinitrophenylhydrazine in 30% HClO₄ (11). A control mixture containing compound Z and HClO₄ was incubated under identical conditions. The disappearance of compound Z was monitored during the course of the reaction by chromatography of 20-μl aliquots on HPLC in 5% methanol, pH 2. At the end of the reaction, the phenylhydrazine of compound Z formed in the mixture was visualized by addition of 1 N NaOH to achieve a pH of 11 to produce the characteristic orange color of the hydrazone.

**Quantitation of Phosphorus and Sulfur**—Chemical analysis of phosphate was performed as described by Ames (12). The ratio of sulfur to phosphorus was determined by energy-dispersive x-ray analysis using an ISIS DS-130 scanning electron microscope with a Tracer Northern TN5500 controlling system. Data were accumulated for 99 s with a beam energy of 10 keV and a take-off angle of 45°. Solid samples of thiamine monophosphate and purified compound Z were cemented onto carbon studs with graphite glue. Alternatively, liquid samples of compound Z containing approximately 20–30 μg in 20 μl were applied in 2-μl aliquots and dried onto the carbon studs.

NMR—31P NMR spectra in aqueous solutions were obtained at 25 °C with a General Electric NN 500 spectrometer operating at 121.3 MHz using 10-mm NMR tubes. D₂O in the sample buffer served as a field frequency lock. 31P NMR spectra in dimethyl sulfoxide were acquired with a General Electric GN 500 spectrometer operating at 202.5 MHz. Chemical shifts are referenced to 85% phosphoric acid with downfield chemical shifts being expressed as positive numbers.

**'H NMR Spectra**—In the course of these experiments it was noted that extracts not exposed to oxygen or iodine exhibited a large peak of 300 nm absorbing material eluting at 3.8 min. This material gradually disappeared as compound Z, eluting at 8.5 min, was formed. The apparent inverse relationship raised the possibility that compound Z might be derived from the material eluting at 3.8 min and that the latter could be the molybdopterin precursor in its active form. Assay of HPLC fractions for molybdopterin precursor activity revealed that precursor activity was indeed present in those fractions corresponding to the 3.8-min peak.

**Fast Atom Bombardment Mass Spectroscopy**—Fast atom bombardment mass spectroscopy data were obtained with a VG 70S instrument equipped with an 11 250 data system. Samples were exposed to xenon atoms at 8 keV translational energy. Low resolution spectra of samples were obtained by scanning from m/z = 50 to 600 at 5 s/decade. Accurate masses were measured by peak matching at a resolution of 10,000 (10% valley) using calibration ions of known exact mass in the spectrum of decaenol carnitine as references. A 200-μl sample of compound Z (80 μg) in H₂O was treated with 20 μl of a slurry of Dowex 50WX12 (H⁺ form, 200-400 mesh, Bio-Rad) to remove cations which suppress ionization. The sample was then lyophilized to dryness, dissolved in a few microliters of H₂O, and transferred in 1-μl aliquots to a probe tip layered with a "magic bullet" matrix for analysis.

**RESULTS**

**Purification of Compound Z**—As noted earlier (7), compound Z could be obtained from acid/iodine-treated extracts of *E. coli* chlM and chlN and *N. crassa* nit-1. Although all these mutants produce the same species in good yield, the *E. coli* mutants proved to be better source material for isolation of compound Z in large quantities. To optimize the yield of compound Z, various conditions for cell culture, cell breakage and extraction were explored. It was observed that aerobic and anaerobic cultures of *chlM* and *chlN* produced equivalent amounts of compound Z/g of cell weight. Cells were grown anaerobically for studies involving active molybdopterin precursor; however, for routine purifications of compound Z, aerobic culture conditions were used to increase the cell density. Cultures of *chlM* and *chlN* were used interchangeably throughout these studies.

Direct acidification of a suspension of whole cells in H₂O to pH 1 was found to be an effective strategy for minimizing the time and amount of manipulation required to release the precursor from cells. For preparation of compound Z, the acidified cell suspension was subjected to iodine oxidation, centrifuged, and chromatographed on QAE-Sephadex and Florisil columns as described under "Materials and Methods." This was followed by purification by HPLC in three different solvent systems. In all three solvents, as well as in 50 mM triethylammonium acetate, compound Z isolated using the procedure described above co-eluted with a sample purified by an earlier method (6). Using the modified procedure, approximately 150–200 μg of purified compound Z could be isolated from a 6-liter culture of aerobically grown cells. The purified compound Z was stored as a dry sample or dissolved in H₂O.

**Relationship of Active Precursor to Compound Z**—Early in the course of characterization of compound Z, experiments were performed to establish whether any compound Z is formed in vivo in *E. coli* chlM and chlN cells and whether its production in vitro was absolutely dependent on the presence of iodine as oxidant. For this purpose, acidified, centrifuged extracts of anaerobically grown *chlM* cells were prepared and immediately analyzed by HPLC without iodine oxidation or any other intervening purification steps. Extracts prepared in this manner were devoid of compound Z. However, if extracts were maintained aerobically at acidic pH prior to HPLC, compound Z was indeed formed. The time course of aerobic formation of compound Z was relatively slow, requiring several hours at room temperature to reach completion, but the limit production was equivalent to that obtained when iodine was added to the extracts.

In the course of these experiments it was noted that extracts not exposed to oxygen or iodine exhibited a large peak of 300 nm absorbing material eluting at 3.8 min. This material gradually disappeared as compound Z, eluting at 8.5 min, was formed. The apparent inverse relationship raised the possibility that compound Z might be derived from the material eluting at 3.8 min and that the latter could be the molybdopterin precursor in its active form. Assay of HPLC fractions for molybdopterin precursor activity revealed that precursor activity was indeed present in those fractions corresponding to the 3.8-min peak.

To explore these relationships further, material corresponding to the 3.8-min peak was isolated, and the time courses of disappearance of precursor activity, disappearance of the 3.8-min peak and appearance of compound Z were monitored. The results, shown in Fig. 3, demonstrate a direct correlation between the loss of precursor activity and the decrease in the 3.8-min peak, verifying that this peak corresponds to molybdopterin precursor. From these results it is also apparent that the time-dependent increase in compound Z is coincident with a decrease in the 3.8-min peak, as noted earlier, and with a decrease in active precursor. The lines representing loss of activity and increase in compound Z intersect at 50% indicating that the precursor is oxidized directly to compound Z with no accumulation of stable intermediates. The identification of compound Z as the direct oxidation product of the molybdopterin precursor added considerably to the significance of further structural studies of compound Z since the results obtained would be directly relevant to an understanding of the molybdopterin biosynthetic pathway.

**Absorption Spectra**—The absorption properties of compound Z were reminiscent of those of oxidized sepiapterin (13) and 6-propionylpterin, both of which contain a carbonyl group or C-1’ of the side chain. Fig. 4 compares the absorption spectra of compound Z and 6-propionylpterin at pH 1, 5, 12, and 13. The spectra of the two compounds are strikingly similar at all pH values. At pH 5 and 12, the long wavelength...
Compound Z is very stable between pH 3 and 11, but is degraded at more extreme pH conditions to a number of products which have not been fully characterized. The major degradation product produced in base has been identified as pterin-6-carboxylic acid.

Sodium Borohydride Treatment—Chemical evidence for the presence of a carbonyl function in compound Z was derived from the results of reduction with borohydride. Compound Z in 50 mM ammonium acetate, pH 6.8, was reacted with an excess of sodium borohydride at room temperature. The pH of the reaction mixture was monitored and maintained below 8.5 by the addition of dilute HCl. The course of the reaction was followed by examining aliquots of the mixture by HPLC in 50 mM ammonium acetate, pH 6.8. Initial reduction of compound Z generated two products in equal amounts, eluting slightly earlier than compound Z. These products had absorption spectra identical to each other and suggestive of oxidized pterins, but quite different from the spectrum of compound Z. Upon further incubation with borohydride, these two products were converted to two new products, eluting earlier still, with identical spectra similar to those of 7,8-dihydropterins. After 30 min of borohydride reduction, two stable species with identical spectra typical of tetrahydropterins were formed which were retained only slightly on the HPLC column. As shown in Fig. 5, acidification of the reaction mixture at this stage to pH 2 and oxidation with excess iodine led to regeneration of the two initial products of borohydride treatment with no formation of compound Z. From the spectral properties of the two products (Fig. 5) it was apparent that the initial site of borohydride attack is that function on the side chain of compound Z responsible for the extended conjugation, i.e. a carbonyl on C-1'. Reduction of the carbonyl produces an enantiomeric mixture of two species with hydroxyl functions on C-1' which have identical spectra indistinguishable from those of typical polyhydroxy pterins such as neopterin or bioterpine (14). Further incubation with borohydride results in the sequential reduction of the pterin ring to the 7,8-dihydro and tetrahydro levels; these latter effects are fully reversible by subsequent treatment with iodine yielding oxidized pterin species which retain the hydroxy function on C-1'.
molybdopterin precursor that gives rise to compound Z. Their absence in compound Z could suggest either that they are also absent in the precursor and are incorporated by the chIAl converting factor or that they are present in the precursor but are lost during the oxidative conversion to compound Z.

Chemical analysis of several preparations of compound Z revealed that all contained phosphate. An accurate extinction coefficient of 13,600 ± 540 (S.E.) for compound Z at pH 12 was determined based on a phosphate content of 1.0 mol/mol. This can be compared to a value of 11,482 reported for 6-propionylpterin (15). Quantitation of pterin in compound Z by oxidation with alkaline permanganate to pterin-6-carboxylic acid (2, 3) supported the conclusion that compound Z contains 1 mol of phosphate/mol. The yield of pterin-6-carboxylic acid was consistently 10–15% lower than that predicted from the extinction coefficient of compound Z; however, HPLC analysis of the alkaline permanganate oxidized sample of compound Z revealed the presence of two products. The major product was identified as pterin-6-carboxylic acid; the minor product was not characterized but presumably arose from base-catalyzed degradation in compound Z by an alternative route.

Sulfur was quantitated by energy dispersive x-ray analysis. It is difficult to obtain absolute quantitation by this method, but the ratios of constituent elements can be determined reliably. As shown in Fig. 6, no x-rays of the energy corresponding to sulfur were detected in the sample of purified compound Z although the presence of phosphorus was clearly indicated. Samples of compound Z obtained at various stages during the purification were examined by the same technique. Sulfur was not present in any of the samples, although phosphorus emission was observed in all cases. With the phosphate stoichiometry of compound Z established by an independent assay, it can be concluded from the x-ray analysis data that compound Z does not contain sulfur.

The phosphate group in compound Z is not present in the form of a simple monoester as evidenced by its insensitivity toward alkaline phosphatase digestion (Table I). Evidence for the presence of a phosphodiester or cyclic phosphate function was sought by incubation with phosphodiesterase I, 2',3'-cyclic nucleotide 3'-phosphodiesterase, and 3',5'-cyclic nucleotide phosphodiesterase. None of these treatments resulted in any change in the mobility of compound Z on HPLC or generated a product which was susceptible to alkaline phosphatase. A cyclic phosphate structure analogous to that in neopterin 2':3'-cyclic phosphate was tested for by subjecting compound Z to hydrolysis in 2 M HCl at 100 °C for 2 h. This treatment converts neopterin cyclic phosphate to a monoester derivative (16). However, as indicated in Table I, acid hydrolysis of compound Z for even 1 h led to quantitative release of the phosphate as inorganic phosphate. The results of these experiments did not rule out the presence of a cyclic phosphate in compound Z. Substrate specificities of the diesterases could easily preclude their recognition of compound Z, and the presence of a carbonyl on C-1' and/or a cyclic phosphate different from the 5-membered ring of neopterin cyclic phosphate could account for the aberrant behavior of compound Z when subjected to acid hydrolysis.

**P NMR Spectroscopy**—Direct evidence for a cyclic phosphate in compound Z was provided by 31P NMR spectroscopy. Spectra of compound Z were obtained in D2O, in 15 mM ammonium acetate at pH 5, in 50 mM Tris-HCl at pH 8.4, and in dimethyl sulfoxide. The spectra in the latter two solvents are shown in Fig. 7. In all cases, a resonance at 9 to 3 ppm was observed as a well defined doublet that collapsed to a singlet when broad band proton decoupling was employed. No other resonances were observed over the range of -30 to +200 ppm. The chemical shift of the phosphorus resonance in compound Z is in the range characteristic of a cyclic phosphate in a six-membered ring (17). Moreover, the very minor change in chemical shift from -3.240 at pH 5 to
The assignment of the five remaining resonance lines to protons in the proposed structure is summarized in Table II. The singlet at 9.15 ppm is attributed to the C-7 proton of the pyrazine ring. The chemical shift of this resonance is in the range typical of oxidized pterins (14). Assignment of resonances to the C-4' methylene protons is simplified by first identifying proton-phosphorus couplings in the 31P NMR spectrum. The strong P-H coupling noted in the 31P NMR spectrum has its counterpart in the 1H NMR spectrum in the quartet at 4.15 ppm. Quantitative analysis of the splitting pattern (Table II) reveals that the proton responsible for this signal interacts with the phosphorus nucleus with a coupling of 21.9 Hz and with another proton with a coupling of 11.9 Hz. The proton causing the 11.9 Hz coupling is clearly the one which appears in the 1H NMR spectrum as the doublet at 4.50 ppm. Again, by analogy to adenosine 3':5'-cyclic phosphate, it is reasonable to conclude that the 4.15 ppm quartet and the 4.50 ppm doublet arise from the methylene protons on C-4' of compound Z. One of the protons is fixed in a trans orientation which gives rise to the strong H-P coupling while the other proton, in the gauche orientation, interacts only weakly with the phosphorus nucleus. These two methylene protons are coupled to each other with a coupling constant of 11.9 Hz which can be compared to the J(31P,31P) of 9.7 Hz in adenosine cyclic phosphate (18).

The two remaining resonance lines at 5.99 and 4.47 ppm are apparent singlets which are not exchanged when D2O is added to the sample. If these signals arise from the methine protons on C-2' and C-3', it might be expected that they would display some interaction with each other, with the C-3' hydroxy proton, or with one or both of the C-4' methylene protons. The two-dimensional COSY spectrum (data not shown) revealed strong cross-peaks between the C-4' methylene protons at 4.50 and 4.15 ppm, as expected, and also displayed additional cross-peaks indicative of very weak interactions between the two proton resonances at 5.99 and 4.47 ppm. These interactions of less than 1 Hz are not of sufficient magnitude to split the signals into resolved doublets in the one-dimensional spectrum. In the absence of any observable interactions between the two proton resonances at 5.99 and 4.47 ppm. These interactions of less than 1 Hz are not of sufficient magnitude to split the signals into resolved doublets in the one-dimensional spectrum. In the absence of any observable interactions between the two proton resonances at 5.99 and 4.47 ppm. These interactions of less than 1 Hz are not of sufficient magnitude to split the signals into resolved doublets in the one-dimensional spectrum.
interactions with the hydroxy or methylene protons it is not possible to make a definitive assignment of either the 5.99 or 4.47 ppm resonance specifically to a C-2' or C-3' proton. However, the presence of signals from these two weakly coupled carbon-bound protons is consistent with the structure proposed in Fig. 2.

The one proton in the postulated structure of compound Z which is not accounted for by the 1H NMR results is the C-3' hydroxy proton. The 1H NMR spectrum of another, less concentrated sample of compound Z was analyzed and proved to be fortuitously low in the triplet artifact at 7 ppm. In that spectrum (data not shown) a singlet at 6.97 ppm integrating to one proton was evident which was exchanged with the sodium adduct, respectively, of a compound with this formula. The exact mass of the m/z ion was determined to be 344.0401, a value within 1.4 ppm of the theoretical exact mass for the formula C24H20N6O7P.

**DISCUSSION**

The studies reported here have led to the elucidation of the structure of the oxidized form of the molybdopterin precursor from *E. coli* chlM and chlN and *N. crassa* nit-1 mutants. Although the chlM and chlN loci are located in distinct non-overlapping regions of the *E. coli* chromosome, the gene products of both loci are required for the production of a functional converting factor which acts to convert the precursor to molybdopterin (6, 7). Mutants at either locus are deficient in molybdopterin and dependent enzymes and also accumulate the precursor which is the substrate for the converting factor in the molybdopterin biosynthetic pathway. A third *E. coli* mutant, chlA1, contains active converting factor but is blocked earlier in the molybdopterin biosynthetic pathway (6, 7). While the genetics of molybdopterin biosynthesis in *N. crassa* have been less well characterized, the phenotype of the nit-1 mutant is analogous to that of chlM and chlN.

From the fact that the chlA1 converting factor is capable of directly converting the precursor from all three sources into molybdopterin, it was expected that the precursor would contain a majority of the features present in molybdopterin. This expectation has been borne out by the finding that compound Z, like molybdopterin, contains a 6-alkyl side chain and a phosphate group. The chemical nature of the phosphate in compound Z proved to be highly unusual. Instead of the simple monoester identified in molybdopterin and its in vitro degradation products, the phosphate in compound Z is bound to C-2' and C-4' of the side chain forming a six-membered cyclic structure. Several pterins bearing cyclic phosphate groups have been reported; however, all species identified to date contain five-membered cyclic structures carried on 3-carbon alkyl side chains (16, 19). If the cyclic phosphate of compound Z is present also in the active precursor, as appears likely from ionic properties and phosphatase insensitivity, it could be envisioned that this novel structure is designed for activation of the C-2' for sulfur addition. In the molybdopterin precursor, energization of the C-2' through cyclic phosphodiester bond formation could facilitate sulfuration in a manner analogous to the formation of cysteine from serine. In the latter case, the β-carbon of serine is activated by O-acetylation prior to sulfur addition.

The fact that the C-2' of compound Z is part of the cyclic phosphate structure abrogates the presence of the C-2' thiol in the precursor, required as a constituent of the endothiol of molybdopterin. The absence of sulfur in compound Z might suggest the absence of sulfur in the precursor. However, it is possible that the active precursor contains a thioketone on C-1' or that the cyclic phosphate in compound Z is present in the precursor as a phosphorothioate. The sulfur in both such structures is unstable (20) and could be replaced by oxygen during the *in vitro* oxidative manipulations.

One observation that bears on the question of whether sulfur is lost in the conversion of active precursor to compound Z is that the latter is generated from active precursor slowly, in the presence of oxygen, with no apparent accumulation of stable intermediates. Since the conversion requires minimally an oxidation of the pyrazine ring, 2 loss of sulfur would necessarily occur as a part of the same overall process. Direct proof that compound Z and active precursor have an

**TABLE II**

| Assignment | Chemical shift (ppm) |
|------------|---------------------|
| ArH (pterin ring, C-7) | 9.12 |
| CH (side chain, C-2' and C-3') | 5.99 |
| CH2 (side chain, C-4') | 4.47 |
| CH2 (side chain, C-4') | 5.00 |
| OH (side chain, 3'-hydroxy) | 6.9? |
| CH3 (side chain, 3'-hydroxy) | 3.4 |

*P* Proton chemical shifts are referenced to tetramethylsilane using residual protons in dimethyl-d8 sulfoxide as an internal standard at 2.49 ppm. The chemical shift of phosphorus is referenced to an external standard of 85% phosphoric acid.

**Fig. 9.** Mass spectrum of compound Z obtained by fast atom bombardment mass spectroscopy. Experimental conditions are described under "Materials and Methods."
identical side chain substituent and differ only in the state of reduction of the pyrazine ring could be afforded by reduction of compound Z back to the active state. Reduction of compound Z with borohydride failed to regenerate active precursor but revealed additional interesting facets of its chemistry. When the course of the reaction of compound Z with borohydride was monitored, it was found that the earliest observed change was reduction of a carbonyl group on the side chain, yielding two isomeric products. The fact that the two products were separable by HPLC was highly significant. Both biotin-terin and neopterin contain two asymmetric centers on the side chains. The resultant stereochecy yields the erythro and three forms of D and L isomers. HPLC of the various stereoisomers has shown that the L- and D-three isomers coelute during HPLC, as do the L- and D-erythro isomers, but the three and erythro forms do not coelute (21). The production of two separable isomers by borohydride treatment of compound Z shows that reduction of the side chain carbonyl to a hydroxyl creates two enantiomeric configurations which in conjunction with a pre-existing fixed asymmetric center generate cis-trans-related isomers. The pre-existing center could be either C-2' or C-3' or both, with C-1' yielding the new asymmetric center.

The studies presented in this article have focused on what appears to be the terminal step of molybdopterin biosynthesis. While certain structural features of the precursor and compound Z provide some insight into earlier steps in the pathway as well. The cyclic phosphate structure of the precursor presumably originates from a cyclase activity on a triphosphate in a manner similar to the action of adenylate cyclase. Were it so, the putative triphosphate is also likely to have a 6-alkyl side chain with a 3-carbon side chain and is the initial pterin-containing precursor of folic acid and of tetrahydrobiotin. It seems likely that the molybdopterin biosynthetic pathway has no common features with the other known pterin biosynthetic pathways. The finding that GTP cyclodrolase deficiency in humans results in biotin deficiency but apparently does not affect molybdopterin formation (22) is in accord with this conclusion.

In recent years a number of patients with an inherited combined deficiency of all molybdoenzymes have been identified (23). Examination of tissues has shown that the genetic defect is in the biosynthesis of molybdopterin in all cases. In studies involving coculture of fibroblasts, it was found that molybdenum cofactor deficient patients may be classified into two groups, with the cells from group B patients secreting a molybdopterin precursor into the medium which the cells from group A patients are able to convert to molybdopterin (24). Recently it was found that urine from group B patients contains a molybdopterin precursor which is functionally identical to the precursor in E. coli and N. crassa mutants and which is converted to compound Z by oxidation (24). These studies show that the active precursor can be transported across membranes, from one cell to another or for excretion in the urine, in intact form. The membrane permeability of the precursor makes it potentially useful in the clinical treatment of group A patients. Beyond that, these findings show that the terminal step in the biosynthesis of molybdopterin is common to man and microorganisms.

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