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FORMATION OF THIENO[3,2-g]PTERINES FROM THE MOLYBDENUM COFACTOR

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A fluorescent oxidation product of the molybdenum cofactor was isolated from *Escherichia coli* nitrate reductase (EC 1.9.6.1) and bovine milk xanthine oxidase (EC 1.2.3.2), which showed a visible absorption band at 395 nm and was dephosphorylated by alkaline phosphatase but not by phosphodiesterase I. The dephosphorylated species was oxidized by periodate to thieno[3,2-g]pterin-2-carbaldehyde which was quantitatively converted to thieno[3,2-g]pterin-2-carboxylic acid by subsequent treatment with Ag_2O in 2 N NaOH. These results indicate that the oxidation product of the molybdenum cofactor is a thieno-[3,2-g]pterin derivative with an unidentified side chain in the 2 position.

Since the existence of a molybdenum cofactor was first reported (1,2), its structure has long been far from understanding, mainly because of its extreme lability in the presence of oxygen (3). Recently, however, some clues to its structure have been presented from several laboratories. In particular, Johnson *et al.* reported the involvement of a novel pterin in the oxidized cofactor (4), although the detailed structure of the side chain and the molybdenum-chelating site still remained to be elucidated. In the course of our studies on multiple fluorescent species obtained from denatured molybdenum enzymes (*Pseudomonas denitrificans* nitrate reductase, *E. coli* nitrate reductase, and bovine milk xanthine oxidase), we observed that some metastable species having a visible absorption band at 395 nm in alkaline solution were formed from the molybdenum cofactor. The isolation from molybdenum enzymes of a similar fluorescent compound (form B) showing an absorption band at 395 nm and containing a phosphate group and a periodate-sensitive function

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was recently reported by Rajagopalan and his associates (5). They reported that this compound and urothione, a sulfur-containing pterin characterized by Goto *et al.*, gave an identical product, 6-carboxypterin-7-sulfonic acid, upon oxidation with alkaline $KMnO_4$, and also that urothione was not detected in the urine samples from molybdenum cofactor-deficient patients. Based on these findings, they suggested a structural relationship between the two molecules.

Recently, we found that the metastable species isolated were gradually decomposed to a common stable form particularily in alkaline solution in the presence of air. We now demonstrate here that the stable form is thieno-[3,2-g]pterin-2-carboxylic acid and that it is quantitatively formed from the metastable oxidation products of the cofactor under controlled conditions.

MATERIALS AND METHODS

Nitrate reductase was purified from *E. coli* according to the procedure of Enoch and Lester (6). Bovine milk xanthine oxidase was purchased from Sigma Chemical Co., St. Louis, Mo. *E. coli* alkaline phosphatase and *Crotalus adamanteus* phosphodiesterase I were purchased from Worthington Chemical Co., Freehold, N.J. Pterin-6-carboxylic acid was obtained by alkaline $KMnO_4$ oxidation of folic acid. 2-Methylthieno[3,2-g]pterin was synthesized from 6-acetonylisoxanthopterin (7) by the procedure described by Goto *et al.* (8). Thieno[3,2-g]pterin-2-carbaldehyde was prepared by cerium(IV) ammonium nitrate oxidation of 2-methylthieno[3,2-g]pterin in 80 % acetic acid (9). Thieno-[3,2-g]pterin-2-carboxylic acid was obtained from thieno[3,2-g]pterin-2-carbaldehyde with Ag_2O in alkaline solution (10,11). Thieno[3,2-g]pterin-2-carboxylic acid was also synthesized by base-catalyzed oxidation of 2-methyl-thieno[3,2-g]pterin (12). Details will be described elswhere. Urothione was isolated from human urine by the procedure described by Goto *et al.* (8). Absorption spectra were obtained on a Union SM-401 spectrophotometer. Fluorescence spectra were measured on a Hitachi 650-10S spectrofluorometer.

RESULTS AND DISCUSSION

We isolated a fluorescent oxidation product of the molybdenum cofactor having an absorption band at 395 nm in alkaline solution from *E. coli* nitrate reductase and bovine milk xanthine oxidase. We referred to this compound as C_{395}. C_{395} and its dephosphorylated form (dp-C_{395}) were purified as follows: Enzyme samples were treated with 30 mM citric acid in 0.5 M NaCl solution and centrifuged. The resulting precipitates were denatured by 6 M guanidine HCl. pH was adjusted to 5.0 with 0.1 N NaOH. After standing for 2 days at room temperature in the dark and in the presence of air, the denatured samples were chromatographed on a florisil column. The bulk of the guanidine was removed

Table I

Thin layer chromatography of oxidation products of the molybdenum cofactor and synthetic compounds

Sample		Rf-values				
		Cellulose				Silica gel G
		A	B	C	D	E
C_{395}*	(I)	0.03	0.02	0.29	0.90	0.33
dp-C_{395}*	(II)	0.21	0.17	0.13	0.15	0.62
after $NaIO_4$ oxidation of II*	(III)	0.23	0.29	0.04	0.06	0.69
after Ag_2O oxidation of III*	(IV)	0.05	0.13	0.05	0.70	0.48
Thieno[3,2-g]pterin-2-carbaldehyde		0.23	0.29	0.04	0.06	0.69
Thieno[3,2-g]pterin-2-carboxylic acid		0.05	0.13	0.05	0.70	0.48
Pterin 6-carboxylic acid		0.12	0.14	0.41	0.90	0.42

*The same Rf-values were obtained with samples from *E. coli* nitrate reductase and from bovine milk xanthine oxidase.

Solvents; A) 1-Propanol : 1% NH_4OH (2:1) B) 1-Butanol : acetic acid : water (4:1:1)
C) 3% NH_4Cl D) Water E) 1-Butanol : 2-propanol : water (10:7:10)

by developing the column with 3 % NH_4Cl. A band of C_{395} showing light-blue fluorescence, which was retarded behind the guanidine, was eluted with distilled water. The samples were further purified by the first and second cellulose column chromatography using 3 % NH_4Cl and distilled water as an eluting agent, respectively. C_{395} thus obtained was chromatographically homogeneous in five solvent systems (Table I). C_{395} was dephosphorylated by alkaline phosphatase but not by phosphodiesterase I, indicating that this compound contains at least one phosphomonoester group. These results well coincide with observation that the activity of the molybdenum cofactor was rapidly lost by treatment with alkaline phosphatase but not with phosphodiesterase I (our unpublished results). Inactivation of the active molybdenum cofactor by alkaline phosphatase was also reported by Hageman *et al.* (13). The alkaline phosphatase-treated C_{395} (dp-C_{395}) was chromatographically identical with a major species which was retained on florisil and eluted by 50 % acetone. dp-C_{395} was purified to homogeneity, by cellulose column chromatography in distilled water. Similarity of the absorption spectra of

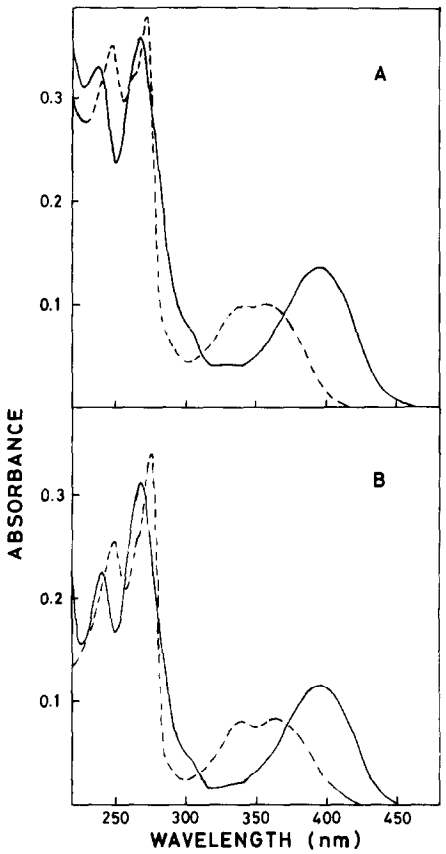

Fig. 1. Absorption spectra of dp-C_{395} from *E. coli* nitrate reductase (A) and 2-methylthieno[3,2-g]pterin (B): ---------, in 0.1 N HCl; ————, in 1 N NH_4OH.

dp-C_{395} to those of synthetic 2-methylthieno[3,2-g]pterin in various conditions suggested that these two are closely related compounds (Fig. 1). Since it is well known that some polyols (14) and thiols (15) are good chelators for molybdate, we tested C_{395} and dp-C_{395} for periodate susceptibility. Although C_{395} itself was not susceptible to $NaIO_4$, dp-C_{395} was converted to a yellowish-green fluorescent species by treatment with aqueous $NaIO_4$ solution. This species was chromatographically indistinguishable with synthetic thieno[3,2-g]pterin-2-carbaldehyde (Table I). It was further oxidized to a more stable compound with Ag_2O in 2 N NaOH, which was purified on a cellulose column. This compound was chromatographically, spectrophotometrically, and fluorometrically identical with synthetic thieno[3,2-g]pterin-2-carboxylic acid (Table I, Fig. 2, and Fig. 3). Now, it is demonstrated that C_{395} is a

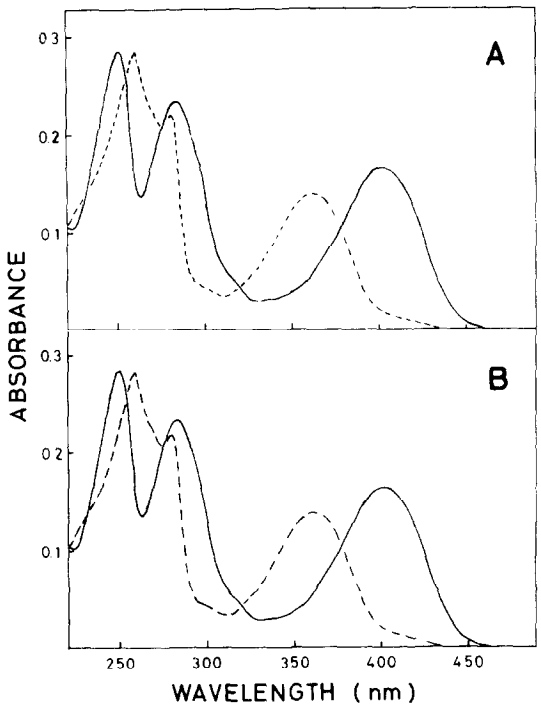

Fig. 2. Absorption spectra of the $NaIO_4$- and Ag_2O-oxidation product of dp-C_{395} from *E. coli* nitrate reductase (A) and synthetic thieno[3,2-g]pterin-2-carboxylic acid (B): --------, in 0.1 N HCl; ————, in 1 N NH_4OH.

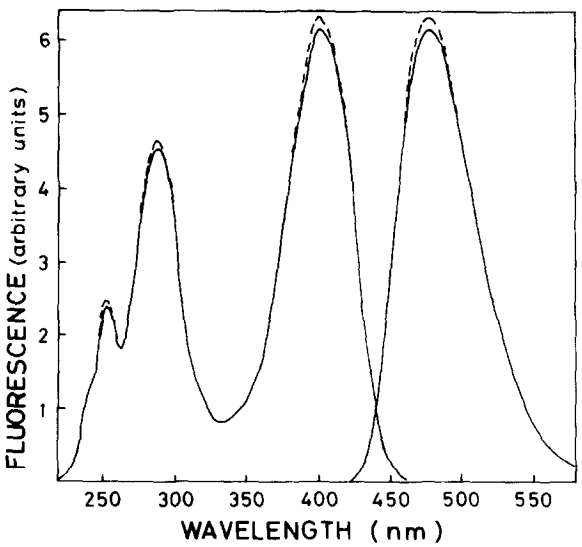

Fig. 3. Fluorescence spectra of the $NaIO_4$- and Ag_2O-oxidation product of dp-C_{395} from *E. coli* nitrate reductase (--------) and synthetic thieno[3,2-g]-pterin-2-carboxylic acid (————) in 1 N NH_4OH.

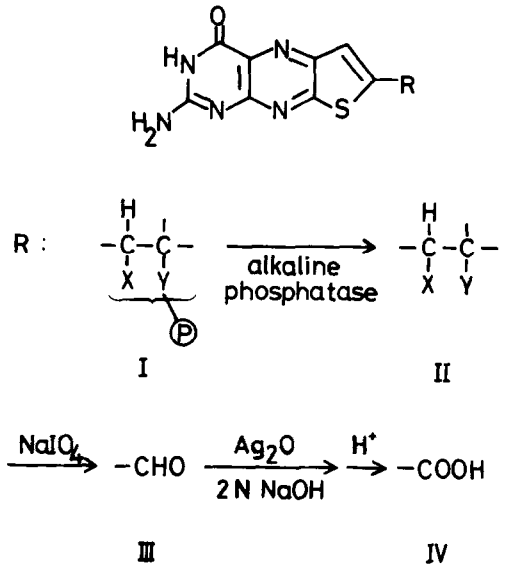

Fig. 4. Possible modification of the side chain in the 2 position of C_{395}.

thieno[3,2-g]pterin derivative with an unidentified side chain in the 2 position. The results described above suggest that the dp-C_{395} has periodate-sensitive vicinal groups on the 1' and 2' carbon atoms of the substituent in the 2 position, which is blocked by a phosphate group in C_{395}. For explanation, possible modifications of the side chain in the 2 position are presented in Fig. 4. In addition to the similarlity of the 2-substituent of C_{395} and dp-C_{395} to that of urothione, thieno[3,2-g]pterin-2-carboxylic acid was also obtained from urothione by some treatment, *e.g.* in alkaline solution in the presence of $NaMoO_4$ and air. These results provide evidence supporting the idea of Rajagopalan and co-workers that the molybdenum cofactor and urothione are structurally related.

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