Purification and Properties of Dihydroorotate Oxidase from *Crithidia fasciculata* and *Trypanosoma brucei*†

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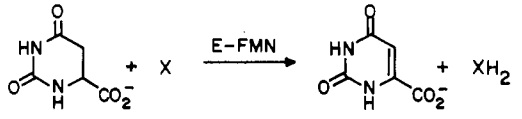
ABSTRACT: Dihydroorotate oxidases have been highly purified from the parasitic protozoa *Crithidia fasciculata* and *Trypanosoma brucei*. The *Crithidia* enzyme was purified 4200-fold from a crude soluble protein extract in four steps. The protein is a dimer as judged from the native (M_r 60 000) and subunit (M_r 32 700) molecular weights. The purified enzyme exhibits a characteristic flavin electronic spectrum, and each mole of native dimer contains 1.0 mol of tightly bound flavin mononucleotide. Under anaerobic conditions, the flavin chromophore is reduced upon addition of L-dihydroorotate. In air-saturated buffer, the enzyme catalyzes the conversion of L-dihydroorotate to orotate with concomitant reduction of equimolar amounts of molecular oxygen to hydrogen peroxide. A variety of low molecular weight oxidants (e.g., quinones or ferricyanide) may replace oxygen as the electron acceptor during catalysis. The dihydroorotate oxidase of *T. brucei* was purified 1400-fold to apparent homogeneity by a highly similar isolation procedure. The estimated native (M_r 62 000) and subunit (M_r 30 500) molecular weights indicated a dimeric protein comparable in size to the enzyme from *Crithidia*. These results suggest that dihydroorotate oxidation is mediated by flavoprotein oxidases in these parasitic protozoa rather than by pterin-linked hydroxylases as recently proposed [Kidder, G. W., & Nolan, L. L. (1973) *Biochem. Biophys. Res. Commun. 53*, 929–936; Gutteridge, W. E., Dave, D., & Richards, W. H. G. (1979) *Biochim. Biophys. Acta 582*, 390–401].

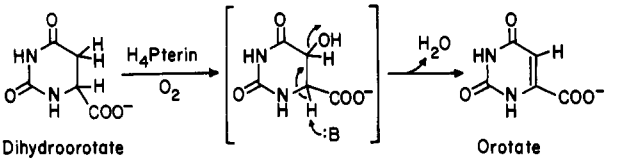
Pyrimidines are key monomer units of nucleic acids, and the selective inhibition of their biosynthesis has been a strategy in the design and development of antitumor, antimicrobial, and potentially antiparasitic agents. One potential point of attack is the conversion of dihydroorotate to orotate.

The enzymes responsible for this transformation in bacteria are flavoproteins of two types. When *Clostridium oroticum* is presented with orotic acid as the sole carbon source, a degradative dihydroorotate dehydrogenase is induced that functions as an orotate reductase at the expense of NADH[1] oxidation (Lieberman & Kornberg, 1953). The dihydroorotate is then opened enzymatically, and the product *N*-carbamylaspartate is meshed into normal metabolism as a carbon and nitrogen source. This degradative enzyme contains FMN, FAD, and non-heme iron (Aleman & Handler, 1967). A dihydroorotate dehydrogenase actually functioning in pyrimidine biosynthesis has been detected and partially purified from *Escherichia coli* cytoplasmic membranes (Karibian & Couchoud, 1974), and a soluble biosynthetic enzyme has been purified from *Lactobacillus bulgaricus* as an FMN-containing dehydrogenase (Taylor et al., 1971). These enzymes can utilize a variety of electron acceptors for dihydroorotate oxidation (eq 1; X may be quinones, dyes, cytochrome *c*, or molecular



oxygen), but the identity of the physiological oxidant is unclear.

By contrast, the mammalian enzyme, purified from liver mitochondrial membranes, is not a flavoprotein but rather has been reported to contain zinc and non-heme iron (Forman & Kennedy, 1978). Most recently, Gutteridge et al. (1979) presented evidence that the dihydroorotate oxidizing activity in several species of *Trypanosoma*, the parasitic protozoa responsible for African sleeping sickness and related diseases in animals and humans, consumed oxygen and was stimulated by pterins. They suggested that in these parasites the enzyme might be a pterin-linked hydroxylase, analogous to phenylalanine hydroxylase, which could catalyze the overall dehydrogenation reaction via hydroxylation (e.g., at C-5 as shown in eq 2) and dehydration steps. Some years earlier, Kidder's



group had suggested that a similar pterin-linked dihydroorotate oxidizing enzyme is present in *Crithidia fasciculata* (Kidder & Nolan, 1973), an insect parasite held to be a close biochemical relative of the trypanosomes infecting mammals.

If these parasites indeed use a unique strategy for the enzymic conversion of dihydroorotate to orotate, such an enzyme might be a promising target for chemotherapeutic attack. Although Gutteridge and colleagues reported a 77-fold partial purification of the *Trypanosoma cruzi* enzyme in their study, we felt it necessary to examine these mechanistic proposals with homogeneous enzyme. In this paper, we detail the purification of both the *Trypanosoma brucei* and *Crithidia fasciculata* enzymes several-thousand-fold to homogeneity and report that they are flavin-dependent oxidases.

Materials and Methods

General. L-Dihydroorotic acid, orotic acid, FMN, FAD, pyridine nucleotides, cytochrome *c*, Hepes [*N*-(2-hydroxy-

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[1] Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-cyclohexylethanesulfonic acid; BSA, bovine serum albumin.

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ethyl)piperazine-*N'*-2-ethanesulfonic acid), CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], hemin, streptomycin sulfate, PMSF (phenylmethanesulfonyl fluoride), *p*-iodonitrotetrazolium violet, and 2,6-dichlorophenolindophenol were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) was purchased from Whatman. Matrex gels Orange A and Blue A, and the Orange A monomer were obtained from Amicon. DEAE-Sephacel and Sephadex G-150 were Pharmacia products. Cibacron Blue F3GA was purchased from Pierce Chemical Co. Suramin was obtained from Mobay Chemical Corp. Salicylhydroxamic acid was from Aldrich Chemical Co. Coenzyme Q_0 (2,3-dimethoxy-5-methylbenzoquinone) was purchased from Trans World Chemicals. Electrophoretic reagents were purchased from Bio-Rad Laboratories. All other chemicals were of analytical reagent grade or the highest quality commercially available.

Crithidia fasciculata was grown at 26 °C in 16-L carboys through which a slow stream of pure oxygen was bubbled. The medium contained 1.5% sucrose, 0.5% yeast extract, 0.4% Bacto-Tryptone (Difco), 0.02% streptomycin sulfate, and 0.0002% hemin. Triethanolamine (2.5 mL/L) was added as a buffer, and the initial pH was adjusted to 8.0. The organisms were harvested after reaching a cell density of about 2×10^8 mL^{-1}. The cells were harvested by centrifugation at 3000*g* for 5 min, and they were stored at −78 °C until use.

Trypanosoma brucei brucei (EATRO 110) cells were obtained in large quantities by growth in Sprague-Dawley rats. The animals, approximately 250 g each, were infected by intraperitoneal injection of $(5–10) \times 10^6$ cells. The animals were sacrificed 70–75 h after infection, and the blood was collected by cardiac puncture. The trypanosomes were isolated by differential centrifugation and chromatography on DE-52 according to Lanham & Godfrey (1970). The cells were washed with 50 mM potassium phosphate, pH 7.8, containing 1% glucose, and they were stored at −78 °C.

Protein Determination. Unless otherwise indicated, protein concentrations were estimated by the method of Bradford (1976) with the reagents and standards supplied by Bio-Rad Laboratories. The biuret assay of Goa (1953) was used occasionally in the early stages of protein purification. Estimation of protein concentration by measurement of A_{280}/A_{260} (Kalckar, 1947) was useful for dilute samples of highly purified dihydroorotate oxidase; this method yielded values 10–20% lower than the Bradford assay on identical samples.

Enzyme Assays. Enzyme activity was determined spectrophotometrically by following orotate production at 278 nm. The standard assay mixture (1 mL) contained 100 mM sodium pyrophosphate (pH 9.0), an appropriate amount of enzyme, and 40 nmol of L-dihydroorotic acid. The assay was conducted at 37 °C, and the reaction was initiated by addition of dihydroorotate. At pH 9.0 the extinction coefficient for orotate at 278 nm is 6900 M^{-1} cm^{-1}. For the purification of the trypanosomal dihydroorotate oxidase, the enzyme assay was performed at 37 °C in 5 mM potassium phosphate buffer (pH 7.8). At this pH orotate has an extinction coefficient of 7600 M^{-1} cm^{-1}. One unit of enzyme activity corresponds to the oxidation of 1 μmol of L-dihydroorotate/min at 37 °C. Specific activities are reported as milliunits per milligram of protein.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on a Bio-Rad Laboratories Protean 16 CM apparatus. Slab gels 0.15 cm in thickness were prepared with stacking gels (~1.5-cm long) containing 6% polyacrylamide and separating gels (~12.5-cm long) containing 10%, 11%, or 14% polyacrylamide. Electrophoresis was carried out at a constant 120 V (~30 mA) in the discontinuous buffer system of Laemmli (1970). Prio to electrophoresis, the samples were incubated at 100 °C for 10 min in a solution containing 0.5% $NaDodSO_4$ and 5% 2-mercaptoethanol. Gels were stained with the preparation of Vesterberg (1971), and they were destained with acetic acid–ethanol–water (15:20:165 by volume).

Analytical Gel Filtration. Gel filtration of highly purified *Crithidia* dihydroorotate oxidase was performed on a 77 × 1.2 cm column of Sephadex G-150 equilibrated with 20 mM sodium phosphate (pH 7.4). The column was calibrated by separate chromatographic runs with protein standards [horseradish peroxidase, 40 000; bovine serum albumin, 68 000; *E. coli* alkaline phosphatase, 75 000–80 000; yeast alcohol dehydrogenase, 141 000; rabbit muscle aldolase, 140 000–150 000; bovine liver catalase, 230 000–250 000; phosphorylase *a*, 370 000 (void volume); Pharmacia BD 2000 blue dextran (void volume)], and the molecular weight of dihydroorotate oxidase was estimated by a standard method (Andrews, 1964).

High-Performance Gel Permeation Chromatography (HPGPC). HPGPC was performed in a Waters I-125 column on a Hewlett-Packard 1084B liquid chromatograph. Chromatography was carried out in 50 mM potassium phosphate (pH 7.0). The flow rate was 0.5 mL/min, and detection was at 280 with 600 nm as a reference. Molecular weights were estimated by a method similar to that used for ordinary analytical gel filtration.

Isoelectric Focusing. Isoelectric focusing experiments were carried out on prepoured LKB ampholine PAG plates (pH 4.0–6.5) on an LKB Multiphor apparatus. Electrophoresis was carried out at 4 °C for 1.5 h at 18 W. The gels were stained with Coomassie blue according to a procedure supplied by LKB.

Flavin Determination. Flavin was released from the purified enzyme by extraction of the enzyme solution (in 25 mM Hepes, pH 7.4) twice with an equal volume of methanol. The combined extracts were concentrated under argon in the dark. The identity of the extracted flavin was determined by the HPLC method of Light et al. (1980) with FMN and FAD as standards.

Spectrophotometric and Polarimetric Determinations. Ultraviolet–visible spectroscopy was performed on a Perkin-Elmer Model 554 spectrophotometer. Quartz cuvettes (1-mL volume, 1-cm optical path) were employed for all samples. Oxygen concentrations were measured on a Yellow Springs Instruments Model 53 biological oxygen monitor equipped with a 1-mL cell.

Purification of Dihydroorotate Oxidase from C. fasciculata. Step I: Crude Extracts. C. fasciculata (225 g of packed cells) was mixed with 200 mM Hepes, pH 8.0 (350 mL), and PMSF was added to a concentration of 0.1 mM. After two cycles of freezing (liquid nitrogen) and thawing (30–40 °C), the mixture was centrifuged at 40000*g* for 1 h. The supernatant solution (445 mL) was designated the crude extract.

Step II: Acid Treatment. Orotic acid (5 mL of a 1 mM solution) was added to the crude extract, and the pH was adjusted to 4.1 with concentrated HCl. The resulting mixture was centrifuged at 6000*g* for 5 min; then, the supernatant solution was adjusted to pH 7.4 with 50% NaOH. This solution was dialyzed against 12.5 mM Hepes, pH 7.4 (13 L), for 4 h. The now cloudy solution was centrifuged at 30000*g* for 40 min, and the supernatant was carried on.

Step III: DE-52 Column Chromatography. The acid-treated extract was applied to a column of DE-52 (16 × 2.5 cm). The column was washed with 120 mL of 25 mM Hepes,

Table I: Summary of Purification of Dihydroorotate Oxidase from *Crithidia fasciculata*

purification step	total protein (mg)	total act.[a] (milliunits)	sp act. (milliunits/mg)	x-fold purification	yield (%)
I. crude	11200[b]	10500	0.94	(1)	(100)
II. acid treatment	3280[b]	8260	2.52	2.7	79
III. DE-52 column chromatography	512[b]	5340	10.4	11	52
IV. Matrex Orange A column chromatography	23.7[c]	2790	117	126	27
V. Matrex Blue A column chromatography	0.39[c]	1550	3990	4250	15

[a] Determined at 37 °C in 100 mM sodium pyrophosphate, pH 9.0. [b] Biuret protein assay. [c] Bradford protein assay.

Table II: Summary of Purification of Dihydroorotate Oxidase from *Trypanosoma brucei*

purification step	total protein[a] (mg)	total act.[b] (milliunits)	sp act. (milliunits/mg)	x-fold purification	yield (%)
I. crude extract	1250	561	0.45	(1)	(100)
II. streptomycin sulfate treatment	760	900	1.18	2.6	160
III. ammonium sulfate fractionation	546	434	0.79	1.8	77
IV. DEAE-Sephacel column chromatography	39.0	250	6.4	14	44
V. Matrex Orange A column chromatography	1.24	123	99	220	22
VI. Matrex Blue A column chromatography	0.06	38	630	1400	7

[a] Bradford protein assay. [b] Determined at 37 °C in 5 mM sodium phosphate, pH 7.8.

pH 7.4, and then it was eluted with a 0–250 mM linear gradient of KCl in the same buffer (400 mL). Fractions of 10.4 mL were collected during the gradient elution. The column profile is illustrated in Figure 1. The contents of fractions 15–19 were pooled and dialyzed against 25 mM Hepes, pH 7.0 (1000 mL), for 24 h (one change of buffer), and this material was carried on.

Step IV: *Matrex Orange A Column Chromatography*. The material from step III was applied to a column of Matrex Orange A (17 × 1.5 cm). The column was washed with 100 mL of 25 mM Hepes, pH 7.0, and then it was eluted with 150 mL of 200 mM KCl in the same buffer. Fractions of 5.2 mL were collected throughout; the column profile appears in Figure 1. The contents of fractions 34–35 were pooled and dialyzed against 25 mM Hepes, pH 7.4 (250 mL), for 24 h (one change of buffer).

Step V: *Matrex Blue A Column Chromatography*. The material from Step IV (10.4 mL) was concentrated to 2 mL with a Millipore immersible CX-10 ultrafiltration unit. The concentrated solution was passed through a column of Matrex Blue A (14.5 × 0.7 cm). Fractions of 1.5 mL were collected, and fractions 4–5 contained pure dihydroorotate oxidase. This material was stored at 77 K. Essentially all other proteins from step IV bound to this column, and they were eluted with 1 M ammonium sulfate and 6 M urea (Figure 1). A summary of the purification procedure is given in Table I.

Purification of Dihydroorotate Oxidase from T. brucei. *Step I*: *Crude Extracts*. *T. brucei* (35 g wet weight) was suspended in 5 mM potassium phosphate buffer, pH 7.8 (150 mL), containing 1.6 mM PMSF, and the mixture was subjected to three cycles of freezing and thawing. This material was centrifuged at 9000*g* for 1 h, and the supernatant solution (180 mL) was designated the crude extract.

Step II: *Streptomycin Sulfate Treatment*. Streptomycin sulfate (a 0.15 M aqueous solution) was added to the crude extract to a concentration of 10 mM. The pH was adjusted to 7, and the precipitate was eliminated by centrifugation at 9000*g* for 1 h. The supernatant solution was carried on to the next step.

Step III: *Ammonium Sulfate Fractionation*. Solid ammonium sulfate was added to the enzyme solution from step II to give a final concentration of 40% saturation. The precipitated proteins were eliminated by centrifugation at 9000*g* for 30 min. More ammonium sulfate was added to the supernatant solution to the point of 75% saturation. The precipitate was collected by centrifugation as before, and it was redissolved in 25 mL of 5 mM potassium phosphate, pH 7.8. This solution was dialyzed against 1 L of the same buffer for 12 h (one change of buffer).

Step IV: *DEAE-Sephacel Column Chromatography*. The enzyme solution from step III (26 mL) was applied to a column of DEAE-Sephacel (24 × 1 cm). The column was washed with 15 mL of 5 mM potassium phosphate, and then it was eluted with a linear gradient of potassium phosphate (5–200 mM; 100 mL). Fractions of 5 mL were collected throughout. The contents of fractions 15–17 were pooled and dialyzed against 300 mL of 10 mM potassium phosphate (pH 7.0) for 24 h (one change of buffer).

Step V: *Matrex Orange A Column Chromatography*. The enzyme solution from step IV (15 mL) was applied to a column of Matrex Gel Orange A (6 × 1.5 cm). The column was washed with 25 mL of 10 mM potassium phosphate (pH 7.0), and then it was eluted with the same buffer containing 250 mM KCl. Fractions of 2.5 mL were collected throughout. The contents of fractions 21–23 were pooled and dialyzed against 500 mL of 5 mM potassium phosphate, pH 7.8, for 24 h.

Step VI: *Matrex Blue A Column Chromatography*. The enzyme solution from step V was passed through a column of Matrex Blue A (2.3 × 1 cm). Fractions of 2.4 mL were collected, and the pure dihydroorotate oxidase was found in fractions 2–5. The purified enzyme was stored at −78 °C. A summary of the purification procedure is given in Table II.

Results

Dihydroorotate Oxidase from Crithidia fasciculata. (*A*) *Enzyme Purification*. Our initial attempts at the purification of dihydroorotate oxidase from *C. fasciculata* followed the general procedure of Gutteridge et al. (1979) for partial purification of *T. cruzi* dihydroorotate "hydroxylase": acid treatment, DE-52 chromatography, and Sephadex G-150 chromatography. Phosphate and Tris buffers were employed in their scheme, but we observed the oxidase activity to be somewhat unstable in the latter, so Hepes buffer was substituted and used throughout our procedure. The yield of the

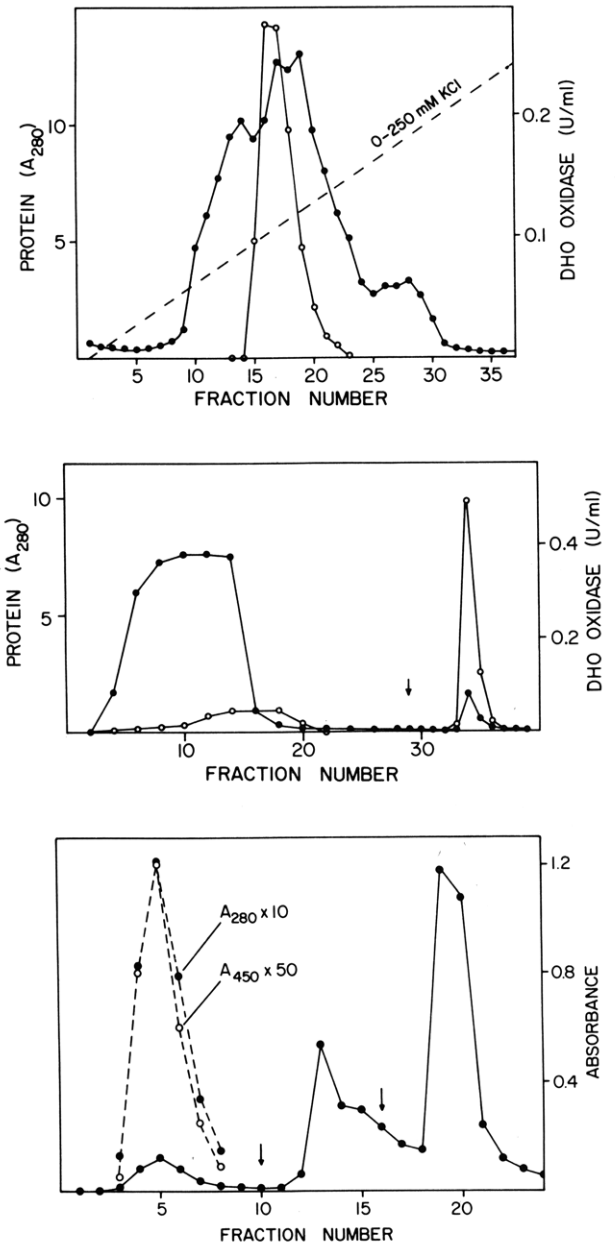

FIGURE 1: Column chromatography of *C. fasciculata* dihydroorotate oxidase. (Top) Elution of the enzyme from DE-52 as described in step III of the purification procedure: (●) protein concentration indicated by absorbance at 280 nm; (○) enzyme activity. (Center) Elution of the enzyme from Matrex gel Orange A as described in step IV of the purification procedure: (●) protein concentration indicated by absorbance at 280 nm; (○) enzyme activity. (Bottom) Elution of the enzyme from Matrex gel Blue A as described in step V of the purification procedure: (●) protein concentration indicated by absorbance at 280 nm; (○) dihydroorotate oxidase detected by its absorbance at 450 nm.

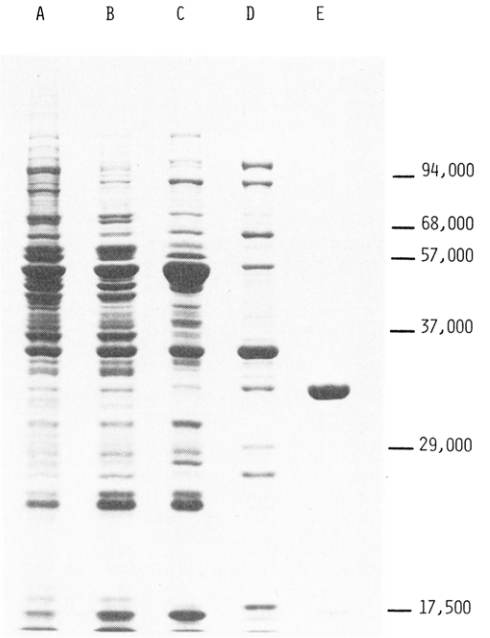

FIGURE 2: $NaDodSO_4$ gel electrophoresis (11% polyacrylamide) of *C. fasciculata* dihydroorotate oxidase after each step of the purification procedure: (A) crude extract (50 μg); (B) after acid treatment (40 μg); (C) after DE-52 column chromatography (30 μg); (D) after Matrex Orange A column chromatography (20 μg); (E) after Matrex Blue A column chromatography (16 μg). The indicated molecular weight standards are rabbit muscle phosphorylase *a* (94 000), bovine serum albumin (68 000), rabbit muscle pyruvate kinase (57 000), yeast alcohol dehydrogenase (37 000), bovine erythrocyte carbonic anhydrase (29 000), and sperm whale myoglobin (17 500).

acid treatment step was significantly improved by the inclusion of 10 μM orotic acid. DE-52 and Sephadex column chromatography gave satisfactory results, but examination of $NaDodSO_4$–polyacrylamide gels of the products indicated that the goal of homogeneous enzyme remained distant.

A breakthrough occurred with the discovery that the oxidase would bind to a column of Matrex gel Orange A, a commercially available triazine dye–agarose chromatography medium. This was quite surprising, since the enzyme had failed to bind to the other dye–agarose gels examined: Matrex gels Red A, Blue A, Blue B, and Green A. Elution of the enzyme from the Orange A column with high salt concentrations yielded a 10–20-fold purification over the DE-52 product. Because the Red A and Blue A gels may be expected to bind 30–60% of the proteins in a random mixture, further purification of the enzyme by filtration of the mixture through one of these media was anticipated. We were surprised and delighted, however, to find that passage of the Orange A product through the Matrex Blue A yielded essentially homogeneous oxidase. As may be seen in Table I and Figure 2, the final combination of dye column chromatography steps effected an amost 400-fold purification over the De-52 step, giving an overall 4200-fold purification to near homogeneity.

One cautionary note must be sounded: *It is essential that all steps from cell breakage to DE-52 column elution be performed as rapidly as possible.* The degree of binding of the enzyme to the Orange A column appears to be inversely proportional to the length of time required for the preceding steps. We suspect that a PMSF-insensitive protease may clip off a fragment of the oxidase that is essential for binding to the Orange A gel but not required for catalytic activity. We are not now able to substantiate this hypothesis due to our inability to purify further the nonbinding species. Its catalytic properties, however, appear to be identical with the Orange A binding oxidase.

The highly purified *Crithidia* dihydroorotate oxidase exhibited a single major band upon $NaDodSO_4$ gel electrophoresis (Figure 2). Gels overloaded with large amounts of protein (25 μg) often showed one minor low molecular weight contaminant (M_r ~17 000), but this was not always present (see, for example, Figure 3). Isoelectric focusing of the purified dihydroorotate oxidase also showed only a single component with a pI = 4.7.

(*B*) *Native and Subunit Molecular Weights.* The native molecular weight of highly purified dihydroorotate oxidase was estimated by two methods. Analytical gel filtration on a column of Sephadex G-150 indicated a native molecular weight

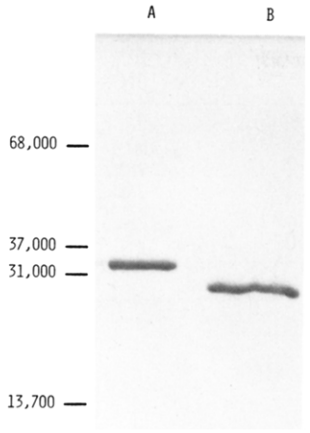

FIGURE 3: $NaDodSO_4$ gel electrophoresis (10% polyacrylamide) of highly purified dihydroorotate oxidases from *C. fasciculata* [(A) 4 μg] and *T. brucei* [(B) 4 μg]. The indicated molecular weight standards are bovine serum albumin (68 000), porcine kidney D-amino acid oxidase (37 000), bovine pancreas deoxyribonuclease I (31 000), and bovine pancreas ribonuclease A (13 700).

of 57 000 ± 3 000 (two determinations). High-performance gel permeation chromatography (HPGPC) on a Waters I-125 column suggested a figure of 63 300 ± 1400 (four determinations). $NaDodSO_4$–polyacrylamide gel electrophoresis of the pure oxidase showed a single band with a molecular weight of 32 700 ± 800 (three determinations on 11% polyacrylamide gels; 10% and 14% polyacrylamide gels gave virtually identical results). These data suggest that *C. fasciculata* dihydroorotate oxidase is a dimer of two subunits of identical molecular weight.

(*C*) *Flavoprotein Nature of the Enzyme.* Solutions of highly purified dihydroorotate oxidase were bright yellow in color. At pH 7.4 the absorption spectrum of the enzyme (Figure 4) exhibited maxima at 274, 368, and 455 nm, indicating the presence of a flavin cofactor. The absorption ratios were $A_{274}:A_{368}:A_{455}$ = 5.71:0.85:1 in our most highly purified preparations.

A sample of the purified protein (70 μg) was extracted with methanol, and the resulting extract was compared with authentic standards of riboflavin, FMN, and FAD by the HPLC method of Light et al. (1980). The extracted flavin was found to have chromatographic properties identical with FMN. If one assumes that the extinction coefficient for the enzyme-bound FMN is the same as that for free FMN at 450 nm (12 200 M^{-1} cm^{-1}), the FMN contents of three different enzyme preparations were estimated to be 0.53, 0.46, and 0.51 mol of FMN/mol of 32 700-dalton subunit, suggesting that each native enzyme molecule contains a single molecule of FMN. There was no significant increase in flavin content or enzyme activity after dialysis of the pure protein against a 10 μM solution of FMN.

The enzyme-bound flavin was reducible with L-dihydroorotate. In a typical experiment (see Figure 4), L-dihydroorotate (50 nmol) was added to a solution of purified dihydroorotate oxidase (~0.9 nmol of flavin, A_{450} = 0.010) in argon-saturated buffer (1.05 mL of 25 mM Hepes, pH 7.4). Spectra were recorded repeatedly until the residual oxygen in the cuvette was consumed with the concomitant production of 34 nmol of orotic acid. At this point, the 450-nm absorption was reduced to 40% of its initial level. Further additions of dihydroorotate resulted in only a slight production of orotate and no further reduction of the flavin chromophore. Subsequent addition of excess sodium dithionite (100 nmol) also failed to further reduce the flavin. However, upon addition of 10 nmol of coenzyme Q_0 (oxidized form) as a mediator of

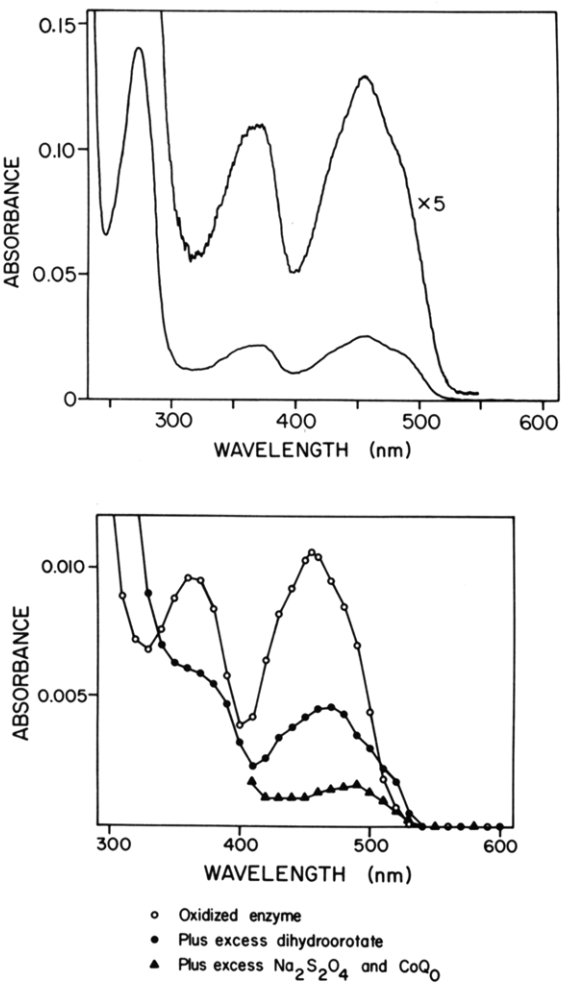

FIGURE 4: (Top) Ultraviolet–visible absorption spectrum of *C. fasciculata* dihydroorotate oxidase. The protein concentration was 0.11 mg/mL in 25 mM Hepes, pH 7.4. (Bottom) Reduction of the flavin chromophore of *C. fasciculata* dihydroorotate oxidase by L-dihydroorotate as described under Results. The protein concentration was approximately 0.06 mg/mL in argon-saturated Hepes, pH 7.4.

reducing equivalents, the 450-nm absorbance was decreased to 11% of its initial value. At no point in this or similar experiments was there any indication of the formation of a long-wavelength absorption (λ >550 nm), though a low, broad band of this type might have gone unobserved due to the very low concentrations of enzyme employed in these experiments (A_{450} never greater than 0.04).

(*D*) *Stability of Enzymatic Activity.* Solutions of the purified dihydroorotate oxidase were stable indefinitely at −78 °C. Storage at 4 °C resulted in an approximately 10% loss of activity per day, but full activity could usually be restored by addition of β-mercaptoethanol or dithiothreitol to a concentration of 0.1 mM.

(*E*) *Optimum pH.* In a series of preliminary measurements, a variety of buffers (phosphate, pyrophosphate, carbonate, Mes, Hepes, Ches, and CAPS) were employed. Considerable variation in enzymatic activity was observed with different buffers at any given pH. Sodium phosphate and sodium pyrophosphate were found to give the greatest activity in the range of pH 6–10; CAPS was the most satisfactory buffer at higher pH. The maximum activity was observed at pH 9.5 in 100 mM sodium pyrophosphate (4.1 μmol min^{-1} mg^{-1}). Note that the standard assay for *Crithidia* dihydroorotate oxidase is conducted at pH 9.0; at this pH the rate of change of absorbance at 278 nm is greatest during a spectrophotometric assay due to variation of the orotic acid extinction

Table III: Utilization of Alternate Electron Acceptors by *Crithidia fasciculata* Dihydroorotate Oxidase

added electron acceptor (50 μM initial concn)	wavelength obsd (nm)	extinction coeff (M^{-1} cm^{-1})	pH	velocity[a] (nmol of $2e^-$/min)
none	278	$\epsilon_{orotate} = 6900$	9.0	1.61
potassium ferricyanide	420	$\epsilon_{ox} = 1000$	9.0	19.0
dichlorophenolindophenol	600	$\epsilon_{ox} = 21\,500$	9.0	0.47
			7.4	4.51
cytochrome *c* + coenzyme Q_0	550	$\Delta\epsilon_{rd-ox} = 19\,100$	9.0	4.93
p-iodonitrotetrazolium violet + coenzyme Q_0	500	$\epsilon_{rd} = 19\,300$	9.0	0.078

[a] See Results for assay conditions.

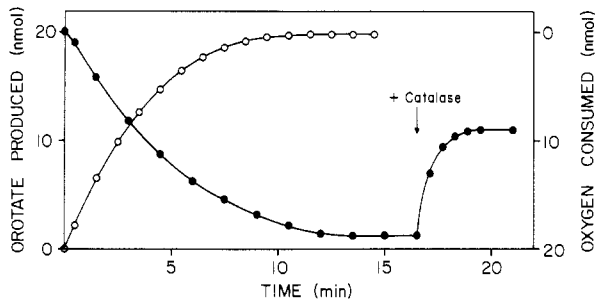

FIGURE 5: Determination of stoichiometry of the reaction catalyzed by *C. fasciculata* dihydroorotate oxidase as described under Results: (○) orotate produced; (●) oxygen consumed.

coefficient with pH. The enzyme was inactive at pH <6, but it retained 60% of the maximal activity at pH 11.

(*F*) *Optimum Temperature.* For assays conducted at pH 9.0, the maximum initial rates for the dihydroorotate oxidase reaction are observed at 42 °C. However, the enzyme is not indefinitely stable to incubation at this temperature, so 37 °C was chosen as the optimum temperature for routine assays.

(*G*) *Stoichiometry of Enzymic Reaction.* Duplicate dihydroorotate oxidase reactions were monitored in the UV–visible spectrophotometer and oxygen electrode. The reactions were carried out at 30 °C and contained 1.8 μg of enzyme, 50 μg of BSA, and 20 nmol of L-dihydroorotate in 1.0 mL of 100 mM sodium pyrophosphate (pH 9.0). Orotate production was monitored at 278 nm ($\epsilon_{pH9} = 6900$ M^{-1} cm^{-1}); and oxygen concentration was monitored polarimetrically. After the reaction had gone to completion, 5 μg of catalase was added. A typical experiment is plotted in Figure 5. For each mole of orotate produced, 0.94 mol of oxygen was consumed. Addition of catalase restored half of the consumed oxygen, indicating that hydrogen peroxide was the ultimate product of oxygen reduction.

(*H*) *Kinetic Properties and Inhibition Studies.* The apparent K_M for L-dihydroorotate was estimated both from measurement of the dihydroorotate concentration at half-maximal velocity during assays carried to completion from low initial substrate concentrations (<5 μM) and from double-reciprocal plots of the initial rates at higher substrate concentrations (>5 μM). At pH 9.0, the dihydroorotate K_M is about 1 μM; at pH 7.4, it is approximately 0.5 μM. At 37 °C and pH 9.0, V_{max} = 4.0 μmol min^{-1} mg^{-1} for our best enzyme preparations. All assays were carried out in air-saturated buffer; the K_M for oxygen was not determined.

Orotic acid is a fairly powerful mixed inhibitor of *Crithidia* dihydroorotate oxidase. The results of a product inhibition study using orotic acid are plotted in Figure 6. From these data, K_I and K_I' are calculated to be 4.5 μM and 36 μM, respectively, at pH 9.0 and 37 °C.

The trypanocidal drugs suramin and salicylhydroxamic acid were examined as inhibitors of the *Crithidia* dihydroorotate oxidase. Both were weakly inhibitory: at a concentration of

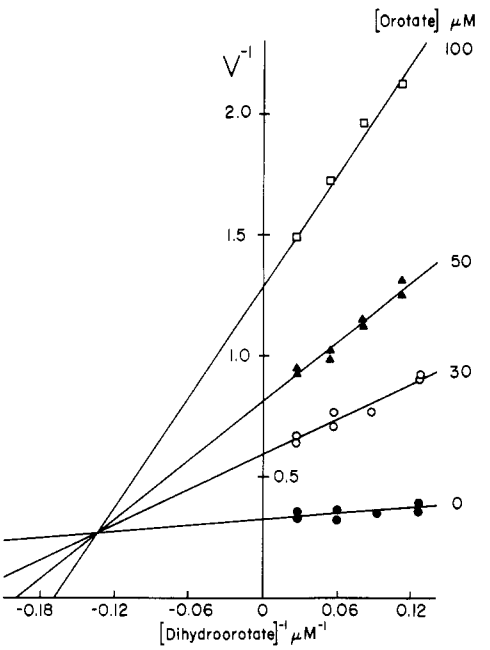

FIGURE 6: Inhibition of *C. fasciculata* dihydroorotate oxidase by orotic acid. All assays contained 0.7 μg of purified oxidase, 50 μg of BSA, 8–40 nmol of L-dihydroorotate, and 0–100 nmol of orotate in a total of 1.0 mL of 100 mM sodium pyrophosphate, pH 9.0. Assays were conducted at 37 °C, and the results are presented in the form of a Lineweaver–Burk plot.

30 μM, V_{max} was reduced by 8% relative to control assays for the former and by 32% for the latter. Assays were carried out at pH 9.0 and 37 °C.

The possibility that the triazine dyes used in the enzyme purification procedure might be inhibitors was also tested. At concentrations of 30 μM, the Matrex Blue A monomer (Cibacron Blue F3GA) and the Matrex Orange A monomer lowered V_{max} by 35% and 23%, respectively, at pH 9.0 and 37 °C.

(*I*) *Alternate Electron Acceptors for Dihydroorotate Oxidase.* Dihydroorotate oxidase can utilize a variety of alternate electron acceptors for the oxidation of dihydroorotate. Table III summarizes the results of a series of assays conducted in the presence of various alternate oxidants. All assays contained 0.7 μg of the purified oxidase, 40 nmol of dihydroorotate, 50 nmol of the added electron acceptor, and 50 μg of BSA in 1.0 mL of 100 mM sodium pyrophosphate (pH 9.0) or 100 mM sodium phosphate (pH 7.4). The buffers were air saturated, so competition from oxygen must be considered in any interpretation. As before, the assays were carried out at 37 °C. Potassium ferricyanide and dichlorophenol were reduced by the enzyme, but cytochome *c* and *p*-iodonitrotetrazolium violet required coenzyme Q_0 as a mediator. The quinone mediator had no effect on the rate of reduction of ferricyanide or dichlorophenolindophenol. Potassium ferricyanide was the most efficient electron acceptor: in the presence of ferricyanide the

rate of catalysis was 12 times faster than with air alone.

Dihydroorotate Oxidase from T. brucei. (*A*) *Enzyme Purification.* A modification of the *Crithidia* dihydroorotate oxidase purification procedure was used to obtain homogeneous enzyme from *T. brucei*. Because of the great time and effort required to prepare large quantities of *T. brucei* protein extracts, we felt it prudent to substitute milder procedures in the early stages of the trypanosomal enzyme purification than were employed for the preparation of the *Crithidia* enzyme. All modifications were first tested with *Crithidia* protein extracts, and homogeneous *Crithidia* enzyme has been prepared by the *T. brucei* purification procedure. Potassium phosphate buffer was employed instead of Hepes buffer throughout the purification, streptomycin sulfate and ammonium sulfate precipitations replaced the acid treatment step, and DEAE-Sephacel was used in place of DE-52. The crucial Matrex Orange A and Matrex Blue A column chromatography steps were essentially unchanged. An overall 1400-fold purification of the trypanosomal enzyme was achieved, yielding material showing one band on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3).

(*B*) *Native and Subunit Molecular Weights.* The native molecular weight of homogeneous *T. brucei* dihydroorotate oxidase was estimated to be 62 000 (one determination) by HPGPC on a Waters I-125 column. The subunit molecular weight was judged to be 30 500 (two determinations) by the results of $NaDodSO_4$ gel electrophoresis. Thus the trypanosomal oxidase also appears to be a dimer of subunits of identical molecular weight.

(*C*) *Optimum pH.* Maximal activity of the trypanosomal dihydroorotate oxidase was observed at pH 9.4 in 5 mM sodium pyrophosphate buffer. At 37 °C the specific activity was 1.4 μmol min^{-1} (mg of protein)$^{-1}$.

Discussion

Our initial interest in the dihydroorotate oxidizing enzymes of *Crithidia fasciculata* and *T. brucei* was prompted by the reports (Gutteridge et al., 1979; Kidder & Nolan, 1973) that these activities were dependent on oxygen and reduced pteridine cofactors. Upon examination of this enzymatic activity in crude acid-treated extracts of *T. brucei*, we readily verified that oxygen was required for dihydroorotate oxidation. However, in variance with the earlier reports, neither tetrahydrobiopterin nor biopterin plus reduced pyridine nucleotides had any influence on the rate of orotate production.

Gutteridge et al. (1979) had reported a 72-fold purification of the dihydroorotate oxidizing enzyme from homogenates of *T. cruzi*. Their product reportedly exhibited "two protein bands" on polyacrylamide gel electrophoresis. When we employed their procedure for our initial purification of the enzyme from *T. brucei* and *C. fasciculata*, it soon became evident that homogeneous protein would only be obtained by much further purification. Concentrating our efforts on the enzyme from the readily cultured *C. fasciculata*, we soon succeeded in achieving a 4200-fold purification to apparent homogeneity. The latter stages of our purification procedure employed two commercially available coupled triazine dye–agarose chromatography media that together permitted a remarkably selective purification of the dihydroorotate oxidizing enzyme.

The highly purified enzyme from *C. fasciculata* exhibited a single protein band upon $NaDodSO_4$–polyacrylamide gel electrophoresis and upon isoelectric focusing (p*I* = 4.7). The molecular weight of the native enzyme was estimated by two forms of molecular exclusion chromatography to be approximately 60 000. A subunit molecular weight of 32 700 was estimated from $NaDodSO_4$ gel electrophoresis; thus the native enzyme is a dimer. The isolated protein is bright yellow, and the electronic absorption spectrum is characteristic of a flavoprotein. On the basis of spectral data and analyses by HPLC, we concluded that each native enzyme contains one molecule of FMN. This flavin chromophore was reduced by the addition of L-dihydroorotate under anaerobic conditions. Furthermore, the stoichiometry of the reaction catalyzed by this enzyme is that of a simple flavoprotein oxidase: L-dihydroorotate and molecular oxygen were consumed in equimolar amounts; orotate and hydrogen peroxide were the products of catalysis. Pterins and pyridine nucleotides appear to have no role in the catalytic mechanism, and we have no ready explanation for the previously reported dependence of enzyme activity on reduced pterins. Our best preparations of *C. fasciculata* dihydroorotate oxidase produced 4.0 μmol of orotate min^{-1} (mg of protein)$^{-1}$ in the presence of saturating L-dihydroorotate in air-saturated buffer at pH 9.0 and 37 °C. This corresponds to a turnover number of 240 min^{-1}, a typical value for a flavoprotein oxidase. The enzyme was also able to efficiently utilize ferricyanide and dichlorophenolindophenol as alternate electron acceptors, and cytochrome *c* was also reduced by the enzyme during dihydroorotate oxidation if coenzyme Q_0 was included to mediate electrons.

The dihydroorotate oxidase of *T. brucei* was purified 1400-fold by a simple modification of the procedure used for the *C. fasciculata* enzyme. The triazine dye–agarose columns again were highly descriminating, and the product enzyme was homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. The native and subunit molecular weights were estimated to be 62 000 and 30 500, respectively, so that the trypanosomal enzyme is comparable in size to its *Crithidia* counterpart. Because only a small amount of the trypanosomal protein was available, we were unable to directly verify the presence of a flavin cofactor in the enzyme.

A comparison of the dihydroorotate oxidases from the two parasitic protozoa with other highly purified dihydroorotate dehydrogenases reported in the literature [from *Clostridium oroticum* (Aleman & Handler, 1967), *E. coli* (Karibian & Couchoud, 1974), *Lactobacillus bulgaricus* (Taylor et al., 1971), *Neurospora crassa* (Miller, 1975), and rat liver (Forman & Kennedy, 1978)] reveals a marked similarity between the *Crithidia* enzyme and that of the bacterium *L. bulgaricus*. Both are soluble proteins; they are similar in size (*C. fasciculata*, 60 000 daltons; *L. bulgaricus*, 55 000), cofactor content (one molecule of FMN per native enzyme molecule), and turnover number (240 min^{-1} and 280 min^{-1}, respectively); and both are able to utilize the alternate electron acceptors dichlorophenolindophenol and ferricyanide.

We have demonstrated that the dihydroorotate dehydrogenase of the parasitic protozoa *C. fasciculata* is a flavoprotein oxidase. The dihydroorotate oxidase from *T. brucei* is probably a flavoprotein also, as indeed are most of the dihydroorotate dehydrogenases that have been purified to homogeneity. The earlier reports (Gutteridge et al., 1979; Kidder & Nolan, 1973) that the parasite enzymes were pterin-linked hydroxylases had led us to hope that we might exploit this unique molecular mechanism for the oxidation of dihydroorotate in the design of specific inhibitors of the trypanosomal enzyme. Such compounds might be useful antiparasitic agents. In light of the present work, the design of trypanosome-specific mechanism-based inactivators of this flavoprotein seems to be a more formidable task. However, the mammalian enzyme is reported to be an iron–zinc metalloprotein lacking a flavin cofactor (Forman & Kennedy, 1978). If true, then a careful study of the mechanisms of the

protozoan and mammalian enzymes might permit the future rational design of selective inhibitors of the trypanosomal dihydroorotate oxidase.

Registry No. Dihydroorotate oxidase, 9029-03-2; L-dihydroorotic acid, 5988-19-2; orotic acid, 65-86-1.

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Kinetic and Thermodynamic Analysis of the Control of C3b by the Complement Regulatory Proteins Factors H and I†

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ABSTRACT: The substrate for the complement serine protease factor I (M_r 88 000) is the C3b portion of the bimolecular complex (M_r 326 000) which is composed of C3b and factor H. This complex is in equilibrium with free C3b and factor H. C3b participates in the recognition function and the positive feedback of the alternative complement pathway, and therefore regulation of C3b activity by factors H and I is crucial for control of this pathway. Conversion of C3b to inactive C3b by factor I (EC 3.4.21.45) was found to be accompanied by a marked decrease in the fluorescence of the probe ANS (8-anilino-1-naphthalenesulfonate), and this change in fluorescence was used to monitor continuously the proteolytic reaction. The initial velocities of the reaction between factor I and various concentrations of the C3b,H complex were analyzed by applying the Michaelis–Menten equation. The analysis indicated that the reaction exhibited simple enzyme–substrate behavior, although the substrate is a bimolecular complex in equilibrium with its subunits. The association constant (K_a) for the complex of C3b and factor H was determined by measuring initial velocities at various concentrations of C3b above and below the estimated dissociation constant for the C3b,H complex. All of the initial velocity measurements were then used simultaneously to refine K_m, V_{max}, and K_a by using an iterative process which yielded the best-fit values of all three constants. The apparent activation energy of the rate-determining step was found to be 24 900 cal·mol^{-1}, reflecting the strong temperature dependence of this proteolytic reaction. The K_a for the interaction between C3b and factor H was 1.6×10^6 M^{-1} at 37 °C and 4.4×10^6 M^{-1} at 20 °C. The K_a for the fluid-phase C3b,H complex was compared to the K_a for the cell-bound C3b,H complex measured in other studies. The comparison suggests that recognition of potentially pathogenic microorganisms by human C3b results in a decrease in affinity between factor H and C3b when C3b is bound to these organisms.

Activation of the complement system leads to the generation of fluid-phase and particle-bound C3b (M_r 176 000) which fulfills various functions in complement reactions (Müller-Eberhard & Schreiber, 1980). Nascent C3b exhibits a metastable binding site (Müller-Eberhard et al., 1966) through which it may form an ester or amide bond with surface constituents of biological particles (Law et al., 1979). C3b initiates the positive feedback system of the alternative pathway (Müller-Eberhard & Götze, 1972) by forming a bimolecular, Mg-dependent complex with the proenzyme factor B (M_r 93 000) and by modulating factor B such that it can be cleaved by its activating enzyme factor D (EC 3.4.21.46). The resulting C3 convertase (C3b,Bb) (EC 3.4.21.47) acts on native C3 (M_r 185 000) and by removing the activation peptide C3a (M_r 9000) produces additional metastable C3b. The first C3b molecules are furnished by the initial C3 convertase of the alternative pathway, C3(H_2O),Bb (Pangburn et al., 1981). Instead of C3b, this fluid-phase enzyme contains uncleaved, functionally C3b-like C3. This form of C3 arises by spontaneous hydrolysis of the internal thioester of native C3 (Law et al., 1980; Pangburn & Müller-Eberhard, 1980; Sim et al., 1981; Tack et al., 1980).

C3b and C3(H_2O) and the respective C3 convertases are

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