Alkyl-dihydroxyacetonephosphate Synthase. Presence and Role of Flavin Adenine Dinucleotide

Vet, Edwin C.J.M. de; Hilkes, Yvonne H.A.; Fraaije, Marco; Bosch, Henk van den

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.275.9.6276

Publication date:
2000

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 9, Issue of March 3, pp. 6276–6283, 2000

Printed in U.S.A.

Alkyl-dihydroxyacetonephosphate Synthase

PRESENCE AND ROLE OF FLAVIN ADENINE DINUCLEOTIDE*

(Received for publication, April 12, 1999, and in revised form, November 8, 1999)

Edwin C. J. M. de Vet‡§, Yvonne H. A. Hilkes‡, Marco W. Fraaije¶‖, and Henk van den Bosch‡**

From the ‡Centre for Biomembranes and Lipid Enzymology, Institute for Biomembranes, Utrecht University, 3584 CH Utrecht, The Netherlands and the ¶Department of Genetics and Microbiology, University of Pavia, 27100 Pavia, Italy

Alkyl-dihydroxyacetonephosphate synthase is a peroxisomal enzyme involved in ether lipid synthesis. It catalyzes the exchange of the acyl chain in acyl-dihydroxyacetonephosphate for a long chain fatty alcohol, yielding the first ether linked intermediate, *i.e.* alkyl-dihydroxyacetonephosphate, in the pathway of ether lipid biosynthesis. Although this reaction is not a net redox reaction, the amino acid sequence of the enzyme suggested the presence of a flavin adenine dinucleotide (FAD)-binding domain. In this study we show that alkyl-dihydroxyacetonephosphate synthase contains an essential FAD molecule as cofactor, which is evidenced by fluorescence properties, UV-visible absorption spectra and the observation that the enzyme activity is dependent on the presence of this cofactor in a coupled *in vitro* transcription/translation assay. Furthermore, we could demonstrate that the FAD cofactor directly participates in catalysis. Upon incubation of the enzyme with the substrate palmitoyl-dihydroxyacetonephosphate, the flavin moiety is reduced, indicating that in this initial step the substrate is oxidized. Stopped flow experiments show that the reduction of the flavin moiety is a monophasic process yielding a oxygen stable, reduced enzyme species. Upon addition of hexadecanol to the reduced enzyme species, the flavin moiety is efficiently reoxidized. A hypothetical reaction mechanism is proposed that is consistent with the data in this paper and with previous studies.

Alkyl-dihydroxyacetonephosphate (DHAP)[1] synthase (alkyl-glyceronephosphate synthase; EC 2.5.1.26) is a key enzyme in the biosynthesis of glycero-ether lipids. It catalyzes the exchange of the acyl chain in acyl-DHAP by a long chain fatty alcohol, yielding alkyl-DHAP (Scheme 1). Because in this step the ether linkage is introduced, this enzyme can be considered to catalyze the most characteristic step in ether lipid synthesis. In mammals it is clearly established that the enzyme is located in peroxisomes (1, 2). This is also emphasized by the observation that the enzyme activity as well as the enzyme protein is deficient in human genetic diseases in which peroxisome biogenesis is disturbed (3, 4). The identification of patients with deficiencies in ether lipid biosynthetic enzymes, including alkyl-DHAP synthase, with severe clinical abnormalities shows unambiguously that ether lipids are of central importance in humans (5).

The reaction mechanism of alkyl-DHAP synthase has been studied extensively in the past using partially purified enzyme preparations and crude microsomal fractions. Important features are: both oxygens of the fatty acid are removed during the acyl cleavage (6) and the oxygen for the ether linkage to be formed is donated by the alcohol (7), pointing toward a unique cleavage mechanism. The *pro*-R hydrogen at the C-1 position of the DHAP moiety is exchanged with water during the reaction, and 1 mol of hydrogen is incorporated from water in alkyl-DHAP for each mole of product formed (8–10). No Schiff base was formed during the reaction (11, 12). The kinetic features of the bisubstrate reaction favors a ping-pong mechanism rather than a sequential mechanism (13, 14).

The cDNAs encoding alkyl-DHAP synthase have been cloned from guinea pig (15), man (16), *Caenorhabditis elegans* (17), and *Dictyostelium discoideum* (18). Both mammalian enzymes carry a peroxisomal targeting signal type 2 in N-terminal cleavable presequences, whereas the *C. elegans* and *D. discoideum* homologs contain C-terminal PTS1 sequences. The expression of guinea pig alkyl-DHAP synthase in *Escherichia coli* (15), as well as the raising of specific antibodies against the protein, has enabled further characterization of the enzyme. Although not an integral membrane protein, the enzyme appears to be tightly bound to the inner aspect of the peroxisomal membrane (19, 20). Alkyl-DHAP synthase is present in a heterotrimeric complex with DHAP acyltransferase, the enzyme that provides the substrate for alkyl-DHAP synthase (21, 22).

Recently, based on the crystal structure of the fungal flavoprotein vanillyl-alcohol oxidase (23) and sequence homology analysis, a novel oxidoreductase family sharing a conserved FAD-binding domain was identified (24). Members of this family are typically 500–600 amino acids long and consist of two domains: (i) a conserved FAD-binding domain anchoring the ADP part of the cofactor which is formed by the N- and C termini and (ii) a nonconserved variable domain of about 240 residues. Remarkably, also alkyl-DHAP synthase, not catalyzing a net redox reaction, was considered to be a member of this family on the basis of its primary sequence. Already after the cloning of the cDNA for human alkyl-DHAP synthase we have noted a clear homology between alkyl-DHAP synthase and the FAD containing D-lactate dehydrogenase (cytochrome *c*) precursor from *Saccharomyces cerevisiae* as well as with an uncharacterized open reading frame product of glycolate oxidase subunit D from *E. coli*, which are also members of this novel oxidoreductase family (see Refs. 16 and 24 for alignments

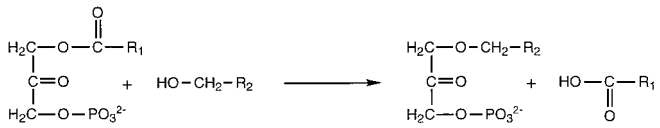
* This work was supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Human Genome Mapping Project Resourse Centre, Hinxton, CB10 1YA Cambridge, UK.

‖ Supported by a fellowship from the European Molecular Biology Organization.

** To whom correspondence should be addressed: Dept. of Biochemistry of Lipids, Centre for Biomembranes and Lipid Enzymology, Padualaan 8, 3584 CH Utrecht, The Netherlands. Tel.: 31-30-2533188; Fax: 31-30-2537990; E-mail: h.vandenbosch@chem.uu.nl.

[1] The abbreviations used are: DHAP, dihydroxyacetonephosphate; FAD, flavin adenine dinucleotide; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis.



SCHEME 1. **Reaction catalyzed by alkyl-DHAP synthase.** R_1 and R_2 represent aliphatic chains.

and references). Mature alkyl-DHAP synthase as well as yeast D-lactate dehydrogenase carry N-terminal extensions of about 100 amino acids that are not shared by other members of the family. Because these two enzymes are the only known membrane-associated proteins of this family so far, these extensions may form membrane anchors. Although existing, examples of FAD-binding enzymes that do not catalyze net redox reactions are rare (25, 26). In this paper we show that alkyl-DHAP synthase does indeed contain FAD and that the flavin part is actively involved in the formation of the product alkyl-DHAP. A reaction mechanism is presented that is consistent with the experimental data presented in this paper and with previous studies (6–14).

EXPERIMENTAL PROCEDURES

Materials

FAD is a product from Roche Molecular Biochemicals. FMN was purchased from Sigma. TnT-coupled reticulocyte lysate system was obtained from Promega (Madison, WI). Amylose resin and the pMAL-c2 vector were obtained from New England Biolabs. Probond™ metal-binding resin was from Invitrogen (San Diego, CA). Isopropyl-β-D-thiogalactopyranoside was a product from Research Organics Inc. (Cleveland, OH).

Methods

Purification of Recombinant Poly-histidine-tagged Guinea Pig Alkyl-DHAP Synthase—Poly-histidine tagged guinea pig alkyl-DHAP synthase was expressed in *E. coli* strain BL21(DE3) as described (15). After the induction period, cells (0.7 liters of culture) were harvested by centrifugation (10 min, 3000 × *g*) and resuspended in 30 ml of ice-cold lysis buffer (20 mM Tris/HCl, 5 mM imidazol, 200 mM NaCl, 0.2% Triton X-100, and 0.2 mg/ml lysozyme, pH 7.5). After 0.5 h of incubation on ice, the suspension was sonicated in two parts for five times for 30 s at 90 Watt output with a macro tip of a Sonifier B12 (Branson Sonic Power Company). All subsequent manipulations were done in a cold room (4 °C). The suspension was centrifuged for 15 min at 12,000 × *g*, and the supernatant was loaded onto a Probond™ metal-binding resin column charged with $NiCl_2$ (1.3 × 6 cm). The flow rate was 30 ml/h. After application of the sample, the column was washed with 50 ml of washing buffer (20 mM Tris/HCl, 100 mM imidazol, 200 mM NaCl, 0.2% Triton X-100, pH 7.5). Recombinant alkyl-DHAP synthase was eluted with a buffer containing 20 mM Tris/HCl, 400 mM imidazol, and 0.2% Triton X-100 (pH 7.5). Because of the relatively low protein concentrations and high Triton X-100 concentration in the samples, the protein concentration was estimated by comparison of staining intensities of the enzyme with known amounts of bovine serum albumin on Coomassie-stained gels.

To determine flavin content of mutant forms of the poly-histidine tagged alkyl-DHAP synthase, proteins were also purified batchwise. For this purpose, 22 ml of isopropyl-β-D-thiogalactopyranoside-induced *E. coli* culture expressing the mutant protein was harvested by centrifugation and lysed in 1 ml of the lysis buffer described above. After clearing of the lysate by centrifugation, the supernatant was added to 150 μl of Probond™ metal-binding resin charged with $NiCl_2$. The recombinant protein was allowed to bind to the material for 0.5 h. The material was washed three to four times with the washing buffer described above, and proteins were eluted with 750 μl of elution buffer.

Fluorescence Detection of Flavin—Fluorescence spectra were recorded with a Photon Technology International Quantum Master fluorimeter with a slit width of 2 nm. Fractions containing purified alkyl-DHAP synthase were denatured with 7 M urea, and excitation and emission scans were recorded as indicated using 1-ml cuvettes. Spectra were routinely corrected for background fluorescence of the buffer. The amount of flavin was quantified by comparing the emission of the sample with the emission of known amounts of reference FAD in the same buffer. For determination of flavin content in mutant forms of the poly-histidine tagged guinea pig protein, 600-μl aliquots of the eluates containing the purified proteins were adjusted to 7 M urea (end volume, 1 ml), and flavin fluorescence was quantified by integration of the emission between 520 and 560 nm (excitation wavelength, 470 nm). The flavin fluorescence was normalized for differences in the concentration of the purified mutant proteins. For this purpose, relative amounts of purified alkyl-DHAP synthase in the eluates were determined by quantification of staining intensities on Coomassie Brilliant Blue stained SDS-PAGE gels with a Bio-Rad GS-700 imaging densitometer.

In Vitro Transcription/Translation—The construct encoding poly-histidine tagged mature guinea pig alkyl-DHAP synthase cloned downstream of the T7 promotor was used (15). The coupled T7 *in vitro* transcription/translation reactions were done according to the manufacturer's instructions (Promega) without radioactive label. The total volume of each reaction was 50 μl, and approximately 1 μg of plasmid DNA was used per reaction. Reactions were performed in the presence or absence of FAD (10 μM) or FMN (10 μM). Formation of the protein was monitored by analyzing an aliquot of 5 μl of the reaction by Western blot immunodetection (19). Aliquots of 10 and 20 μl were used for the determination of alkyl-DHAP synthase activity. Substrate preparation and activity assays were performed as described previously (20) with a slight modification in buffer composition. The following assay conditions were used: 50 mM potassium phosphate (pH 7.5), 50 mM NaF, 5 mM dithiothreitol, 5 mM EDTA, 0.1% Triton X-100, 240 μM palmitoyl-DHAP, and 110 μM [1-^{14}C]hexadecanol (9000 dpm/nmol) in a total volume of 75 μl.

Site-directed Mutagenesis of Histidine 300, Histidine 615, and Histidine 616—The histidine residues at position 300, 615, and 616 in the poly-histidine tagged form of the enzyme were replaced separately by alanines using the Quickchange site-directed mutagenesis method according to the manufacturer's instruction (Stratagene). Incorporation of the mutagenic oligonucleotides into the construct was checked by restriction analysis and nucleotide sequencing. The mutant proteins (denoted as H300A, H615A, and H616A, respectively) were expressed in *E. coli* strain BL21(DE3), and expression levels were found to be comparable with those of the wild type enzyme. Enzyme activies were measured in total cell lysates as described (14). Flavin content was determined by measuring fluorescence as described above.

Construction and Purification of a Maltose-binding Protein-Guinea Pig Alkyl-DHAP Synthase Fusion—The cDNA encoding guinea pig alkyl-DHAP synthase was excised from the pET-15b vector with *Xho*I and ligated into the *Sal*I site of a pMAL-c2 vector. This construct allows the expression of mature guinea pig alkyl-DHAP synthase (amino acids 75–658) as a C-terminal fusion to the maltose-binding protein under control of the tac promotor. This enables the purification of the enzyme in a single step by affinity chromatography (27). The construct was transformed into *E. coli* strain DH5α. An overnight culture (70 ml) was diluted in 700 ml of fresh LB medium containing 50 μg/ml ampicillin, and cells were grown at 37 °C. Cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at an A600 of 0.7 and grown for an additional 3 h. Cells were harvested by centrifugation and resuspended in 20 ml of buffer A (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 2 mM dithiothreitol) supplemented with 0.2 mg/ml lysozyme on ice for 0.5 h. The suspension was sonicated five times for 30 s, and the lysate was cleared by centrifugation (12,000 × *g*, 15 min at 4 °C). The supernatant was applied onto an amylose resin column (volume, 4 ml) with a flow rate of 18 ml/h. The column was washed with four column volumes of buffer A, and thereafter the recombinant MBP-alkyl-DHAP synthase was eluted with 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 2 mM dithiothreitol, 0.2% Triton X-100, and 10 mM maltose. Absorption spectra were recorded on a Hitachi 150-20 spectrophotometer. The concentration of FAD was calculated from the absorption at 450 nm in the presence of 0.5% SDS using 11.3 mM^{-1} cm^{-1} as molar extinction coefficient (28). The substrates palmitoyl-DHAP and hexadecanol were added to the purified enzyme as sonicates in 0.2% Triton X-100.

Stopped Flow Kinetics—Stopped flow kinetics were carried out with a Hi-Tech SF-51 stopped flow apparatus equipped with a Hi-Tech M300 monochromator diode-array detector (Salisbury, UK). All kinetic experiments were performed at 25 °C in 20 mM Tris/HCl (pH 7.5), 2 mM dithiothreitol, 2 mM EDTA, and 0.2% Triton X-100. Spectral scans were collected each 10 ms from 300 to 600 nm. In each experiment 96 scans were collected. Deconvolution analysis of spectral data was performed using the Specfit Global Analysis program version 2.10 (Spectrum Software Assn., Chapel Hill, NC). To determine the rate of enzyme reduction, alkyl-DHAP synthase was mixed with a solution containing 200 μM palmitoyl-DHAP resulting in a final concentration of 100 μM

after mixing. For generation of the reduced enzyme intermediate complex, the enzyme was titrated with an excess of palmitoyl-DHAP (100 μM). By mixing the reduced enzyme species with a solution of 200 μM hexadecanol, reoxidation of the reduced enzyme could be monitored.

Analytical Procedures—SDS-PAGE was done according to Laemmli (29). Protein was determined according to Bradford (30) using bovine serum albumin as standard.

RESULTS

Recombinant poly-histidine tagged alkyl-DHAP synthase was purified in a single purification step to apparent homogeneity from *E. coli* (Fig. 1). To monitor for the presence of flavin fluorescence, an emission scan was recorded (excitation wavelength, 470 nm) from both the native enzyme as well as enzyme denatured in 7 M urea. As can be seen in Fig. 2*A*, a clear fluorescence is observed in case of the denatured enzyme with a maximum of about 525 nm, which is characteristic for flavin. The fluorescence yield is relatively low in the native state, showing that the protein effectively quenches the flavin fluorescence. An excitation scan was obtained with the denatured enzyme (emission wavelength, 530 nm; Fig. 2*B*), which is very characteristic for flavin and resembles the absorption spectrum. The molar ratio between flavin and protein was estimated to be around 0.15–0.25, which shows that only a small part of the enzyme has taken up a flavin cofactor. Nevertheless, because the protein sample appears to be rather pure on SDS-PAGE (Fig. 1, *lane 3*) it is unlikely that this flavin fluorescence is due to another contaminating flavoprotein.

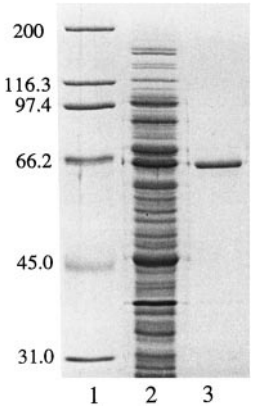


FIG. 1. **One-step purification of poly-histidine tagged guinea pig alkyl-DHAP synthase with affinity chromatography.** *Lane 1*, molecular mass markers (in kDa); *lane 2*, starting material (bacterial supernatant) (3 μl); *lane 3*, pool of fractions containing the purified enzyme (12.5 μl).

In previous *in vitro* transcription/translation experiments (17), we were unable to generate active enzyme despite adequate expression of the protein. If indeed alkyl-DHAP synthase is a FAD-dependent enzyme, this may have been caused by the absence of this particular cofactor. To investigate this, the histidine tagged guinea pig alkyl-DHAP synthase was expressed in a coupled *in vitro* transcription/translation system in the absence or presence of either FAD or FMN (Fig. 3). As can be seen in the Western blot analysis (Fig. 3*B*), the enzyme is expressed to comparable levels in the incubations with no cofactor, with FAD, or with FMN when the DNA template is present. However, only in the FAD-containing incubation does alkyl-DHAP synthase activity become clearly detectable. This provides clear evidence that alkyl-DHAP synthase is dependent on FAD, although it does not catalyze a net redox reaction. It is also shown that FMN cannot replace FAD. This result is in line with the observation that alkyl-DHAP synthase shows homology toward FAD containing enzymes, which contain a conserved domain that specifically interacts with the ADP part of the FAD molecule (24).

In a previous study we prepared single amino acid mutants of the poly-histidine tagged enzyme as well as C-terminal deletion mutants (14). Because histidine may play an important role in the catalytic mechanism (14), we changed in addition all remaining conserved histidines (His^{300}, His^{615}, and His^{616}) into alanines in this study. The inactivity of a given mutant may result from replacement of essential catalytic amino acid residues or from misfolding of the protein, which may be reflected by defective FAD binding. Therefore, we examined for all available mutant enzymes whether flavin content was comparable with the wild type content by assaying batch purified protein for flavin fluorescence. In Table I, the activity and flavin con-

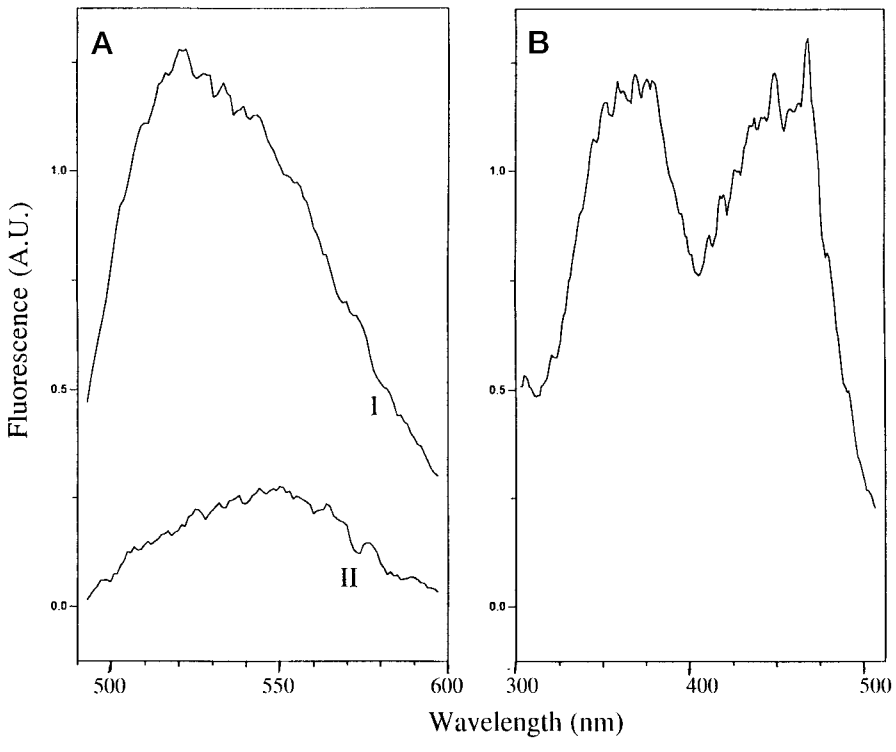


FIG. 2. **Fluorescence of purified recombinant guinea pig alkyl-DHAP synthase.** *A*, emission of the enzyme in the urea-denatured state (*spectrum I*) and the native state (*spectrum II*). Excitation wavelength was 470 nm. *B*, excitation scan of the urea denatured enzyme at emission wavelength of 530 nm.

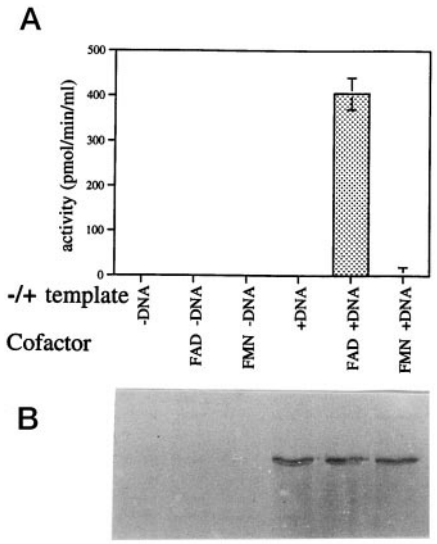


FIG. 3. ***In vitro* transcription/translation of guinea pig alkyl-DHAP synthase in the absence or presence of FAD or FMN.** Reactions were performed without or with DNA template as indicated. The absence of cofactor and the presence of either FAD (10 μM) or FMN (10 μM) in the reaction mixtures is also indicated. *A*, enzyme activities in the reaction mixtures. *B*, Western blot immunostaining of alkyl-DHAP synthase.

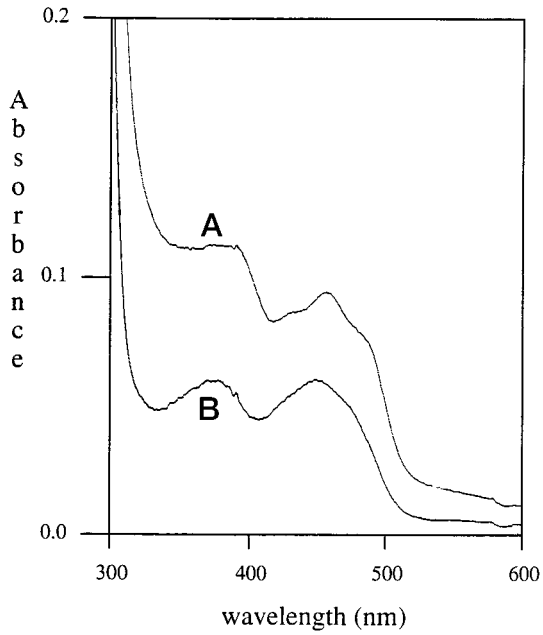


FIG. 4. **Absorption spectra of isolated MBP-alkyl-DHAP synthase in the native state (*A*) or denatured with 0.5% SDS (*B*).**

TABLE I

FAD content and enzyme activities of mutant alkyl-DHAP synthases

Both FAD content (determined by fluorescence) and enzyme activities are expressed as percentages of wild-type values. Values are the means of at least three independent experiments. ND, not detectable (<0.1% of wild type activity). FAD content and enzyme activities of the R419H mutant enzyme are related to the corresponding hybrid wild type construct (pControl as described in Ref. 4). pADSΔ5, the C-terminal 5-amino acid deletion mutant described in Ref. 14.

Mutant	FAD content	Enzyme activity
H300A	14	ND
H615A	56	ND
H616A	135	ND
H617A	157	ND[c]
S367A	47	4[a]
C576A	120	187[a]
R419K	107	1[a]
R419H[b]	123	ND[a]
pADSΔ5	21	ND[a]

[a] *, values derived from previous studies (4,14).

[b] This mutant corresponds to the pMutant construct in Ref. 4 and represents a guinea pig-human hybrid enzyme.

tent of the mutant proteins are given. Certain mutant enzymes have nondetectable activities accompanied by reduced FAD binding (H300A and the deletion mutant pADSΔ5 missing the last five C-terminal residues). Other mutant proteins have nondetectable or strongly reduced activity, whereas flavin content is near normal (R419H, R419K, H616A, and H617A).

Because the poly-histidine tagged protein readily loses activity upon purification, we changed to another affinity tag, *i.e.* the maltose-binding protein. Alkyl-DHAP synthase activities per mg of *E. coli* protein in lysates of cells expressing the MBP-alkyl-DHAP synthase fusion were comparable with the values found for the poly-histidine tagged form (1–3 milliunits/mg) (15). Approximately 60% of the enzyme activity did not bind to the amylose resin affinity column. The enzyme eluted as a yellow protein from the affinity column when a buffer containing 10 mM maltose was applied. About 40% of the applied alkyl-DHAP synthase activity was recovered in the purified fractions. SDS-PAGE analysis showed that the recombinant fusion protein (109 kDa) in the eluate was nearly homogeneous. The purified MBP-alkyl-DHAP synthase contained 0.28–0.30 mol FAD/mol protein. The turnover number of the freshly purified recombinant enzyme in the standard activity assay (20) was determined at 1.0 s^{-1}, using the FAD concentration (derived by either the A_{450} value or by fluorescence measurements) to determine the amount of active holoenzyme. This value corresponds to 900 nmol/min/mg for a 65-kDa protein, which is somewhat higher than the value of 357–640 nmol/min/mg previously determined for the enzyme purified from guinea pig liver (20).This small difference can easily be explained by the lability of the enzyme during the elaborate multi-step purification procedure and inactivation by freeze-thawing during storage.

Fig. 4 shows a UV-visible absorption spectrum of the purified protein, in the native state (*spectrum A*) as well as in the presence of 0.5% SDS (*spectrum B*). The observed spectrum for the native protein is for a flavoprotein displaying two typical flavin absorption maxima at around 375 and 458 nm with shoulders at around 440 and 490 nm. Upon unfolding by SDS, a typical FAD spectrum appears with two unresolved absorption maxima.

To investigate whether the redox state of the flavin cofactor changes because of interaction of the enzyme with the substrate, visible absorption spectra of alkyl-DHAP synthase were recorded in the absence of substrate, in the presence of palmitoyl-DHAP, and in the presence of both palmitoyl-DHAP and hexadecanol. Upon addition of palmitoyl-DHAP, a dramatic change in the visible absorption spectrum is observed (Fig. 5, *spectrum B*). The disappearance of the absorption peaks at 375 and 458 nm is consistent with the FAD cofactor being reduced. Apparently, the substrate acyl-DHAP is oxidized by the enzyme. Inasmuch as these spectra were collected under aerobic atmospheric conditions, we conclude that the reduced flavin in the native enzyme is effectively protected against reoxidation by molecular oxygen from the air. In contrast, when the incubation mixture of enzyme plus palmitoyl-DHAP was treated with 0.5% SDS (conditions as in Fig. 4, *spectrum B*) prior to recording the absorption spectrum, a spectrum of oxidized FAD identical to that shown in Fig. 4 (*spectrum B*) was observed

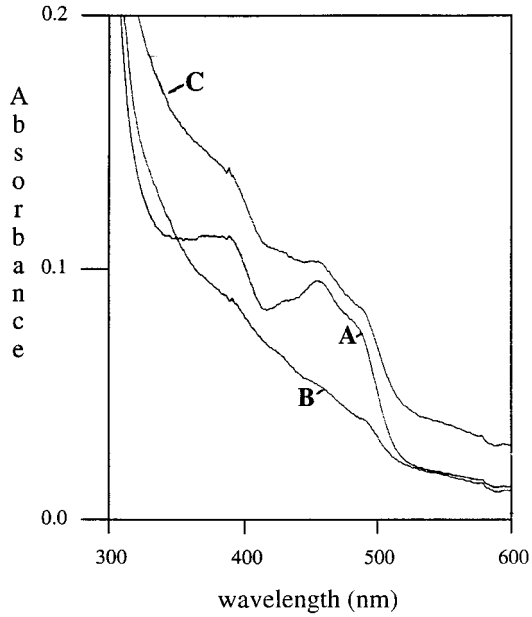


FIG. 5. **Changes in absorption spectra of MBP-alkyl-DHAP synthase as a result of the consecutive addition of the substrates palmitoyl-DHAP and hexadecanol, respectively.** *Spectrum A*, enzyme without substrate added; *spectrum B*, enzyme after the addition of palmitoyl-DHAP (final concentration, 0.1 mM); *spectrum C*, enzyme after addition of hexadecanol (final concentration, 0.15 mM) to the mixture presented in *spectrum B*. Like in all other figures the spectra were recorded under aerobic atmospheric conditions.

(data not shown). This indicates that the reduced $FADH_2$ in the native enzyme becomes readily oxidized by molecular oxygen upon denaturation of the enzyme.

The addition of hexadecanol resulted in a somewhat turbid sample because of the low solubility of this substrate. In routine activity measurements this is prevented by incubating the enzyme with a mixed sonicate of acyl-DHAP and hexadecanol, which gives clear solutions of mixed micelles. However, cloudiness of the samples is an inherent property of the system when the hexadecanol has to be added separately and after the acyl-DHAP, as had to be done in the experiments described in Fig. 5. Even though the spectrum is complicated by light scattering, the reappearance of the characteristic oxidized flavin absorption peaks at around 375 and 458 nm, including the shoulders at around 440 and 490 nm, became clearly visible. Thus, the addition of this second substrate to the acyl-DHAP reduced enzyme appears to reoxidize, at least in part, the flavin (Fig. 5, *spectrum C*).

The reduction of the enzyme upon addition of the palmitoyl-DHAP was studied in more detail using the stopped flow technique (Fig. 6). Upon mixing alkyl-DHAP synthase with palmitoyl-DHAP, a rapid decrease in absorbance around 455 nm indicates that the flavin is efficiently reduced (Fig. 6). Deconvolution of the spectral scans showed that the oxidized enzyme is reduced in a monophasic process at a rate of $0.98 \pm 0.04\ s^{-1}$. From the finally formed spectrum (Fig. 6, *inset*) it can be concluded that the enzyme is effectively reduced by palmitoyl-DHAP, indicating that the reaction with the flavin is essentially irreversible. When spectral changes were followed for a longer period (50 s) no other spectral species could be identified, indicating that the reduced enzyme species is remarkably stable. Stopped flow measurements were also used to monitor the reoxidation of the palmitoyl-DHAP reduced enzyme (data not shown). Although the measurements were hampered by the poor solubility of hexadecanol resulting in perturbation of the spectra (Fig. 5, *spectrum C*), a relative fast reoxidation of the

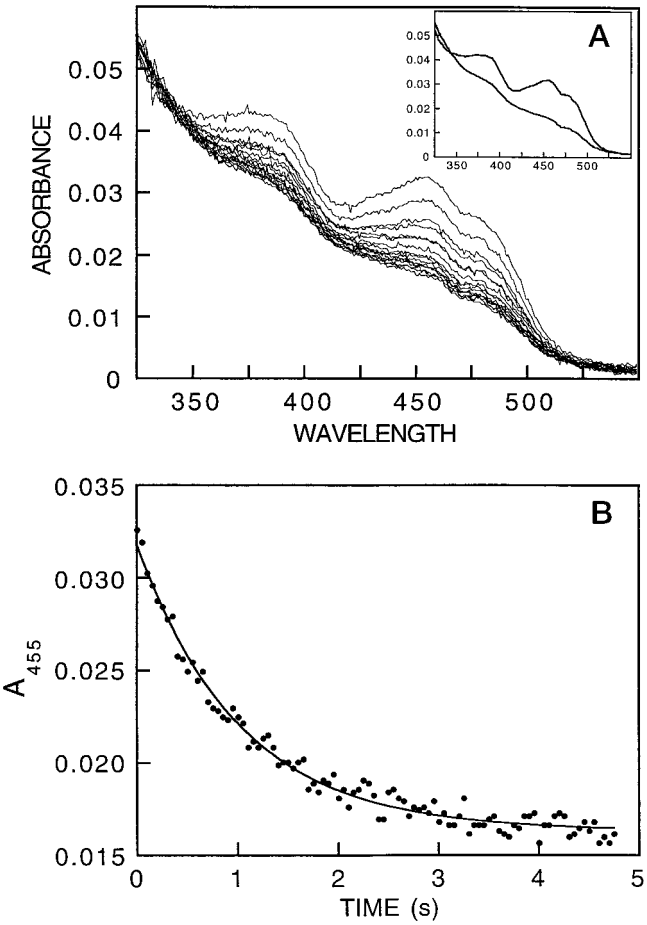


FIG. 6. *A*, spectral changes observed upon mixing of alkyl-DHAP synthase with palmitoyl-DHAP (final concentration, 100 μM). A selection of the original spectral scans is shown (from 6 to 3006 ms with intervals of 200 ms). The *inset* shows spectra obtained after deconvolution of the original spectral scans where the *upper line* represents the initial spectrum of oxidized enzyme and the *lower line* shows the palmitoyl-DHAP reduced alkyl-DHAP synthase species. The spectral data were fit with a one-exponential function ($A \rightarrow B$) resulting in a rate constant of $0.98\ s^{-1}$. *B*, absorbance changes observed upon mixing alkyl-DHAP synthase with palmitoyl-DHAP (final concentration, 100 μM) when monitored at 455 nm. The original data are shown (from 6 to 4756 ms with intervals of 50 ms). The data could satisfactorily be fitted according to a monophasic process using the rate constant ($0.98\ s^{-1}$) obtained by deconvolution of the original spectra.

enzyme could be observed as evidenced by an increase in absorbance between 400 and 500 nm. The estimated rate constant for reoxidation by 200 μM hexadecanol is $15\ s^{-1}$. These data support the data presented in Fig. 5, *i.e.* acyl-DHAP reduced enzyme is reoxidized by hexadecanol.

DISCUSSION

The observation that alkyl-DHAP synthase exhibits homology to FAD containing oxidoreductases (16, 24), especially in the FAD-binding part, led us to investigate a possible role of FAD in this enzyme. Although alkyl-DHAP synthase does not catalyze a net redox reaction, we could convincingly demonstrate the presence of this cofactor associated with the enzyme by fluorescence properties (Fig. 2) and absorption spectra (Fig. 4) of the purified enzyme and by showing that enzyme activity is dependent on this cofactor (Fig. 3). Nevertheless, the recombinant guinea pig enzymes purified from *E. coli* (either as a poly-histidine tagged form or as a MBP fusion protein) did contain less than 1 mol FAD/mol of alkyl-DHAP synthase monomer. This may be due to high expression levels or inefficient

folding. The latter explanation would be in line with the observation that most of the poly-histidine tagged enzyme precipitates as inactive inclusion bodies (15).

It is most likely that the native enzyme, as initially purified in our laboratory from guinea pig liver (20), consists of flavin and protein in a 1:1 molar ratio. However, it is not feasible to obtain the amounts of enzyme required for the studies described in this paper by the elaborate multi-step purification procedure from guinea pig liver. This procedure yielded an enzyme with a turnover number of 0.4–0.7 s^{-1}. When the turnover number for the MBP-alkyl-DHAP synthase fusion enzyme employed in this paper was calculated from activity measurements and using enzyme concentrations deduced from its FAD content, *i.e.* based on the amount of enzyme with a 1:1 molar ratio of flavin and protein, a value of 1.0 s^{-1} was obtained. In view of the difficulties encountered in accurate determination of the protein content of the small amount of the enzyme purified from guinea pig liver (20) and the likelihood of enzyme inactivation during the elaborate purification procedure, we conclude that the turnover numbers are in fact very close. This indeed suggests that the native enzyme very likely contains equimolar amounts of FAD and protein. These considerations then also imply that the recombinant enzyme serves as a good model for the native enzyme, provided its quantity is expressed based on its FAD content.

Inactivity of certain mutant enzymes may also be due to misfolding of the mutant rather than a mere replacement of an active site residue. We reasoned that when the FAD content of such a mutant is clearly decreased compared with the wild type enzyme, the mutation probably affects the structural integrity of the protein. Therefore we investigated the FAD content of all available mutant forms of the poly-histidine tagged guinea pig enzyme (described in Ref. 14 and in this study). The amino acid residues chosen to be mutated are all conserved between the mammalian alkyl-DHAP synthases and the *C. elegans* and *Dictyostelium* homologs. Two mutant forms (H300A and a deletion mutant pADSΔ5 lacking the last five C-terminal residues) clearly exhibit reduced FAD binding (Table I). In line with this finding is that histidine 300 is located in the FAD-binding domain of the enzyme (24) and may be an important structural residue essential for FAD binding. Furthermore, equally in line with these results is the observation that the extreme C-terminal part of the enzyme also constitutes part of the FAD-binding domain (24). The inactivity of these two mutant proteins can best be explained by incorrect folding of the enzyme protein reflected by or caused by a reduced capacity to bind FAD. Certain mutant proteins are inactive but nevertheless exhibit FAD binding capacities comparable with the wild type enzyme (R419H, H616A, and H617A). These mutations probably do not affect the overall tertiary structure of the proteins, and therefore the corresponding mutated residues are more likely to represent bona fide active site residues. In this respect it is noteworthy that His^{615}, His^{616}, and His^{617} are part of a highly conserved stretch of amino acids (amino acids 610–620) that aligns with amino acids 499–509 of vanillyl alcohol oxidase (Swiss-Prot accession number P56216). The latter stretch forms a loop structure containing some active site residues (23), *i.e.* Tyr^{503} and Arg^{504}. In alkyl-DHAP synthase, Arg^{419} is not located in the conserved FAD-binding part but rather in the variable cap-domain. Because there is little sequence homology in this part among the members of this FAD-binding protein family, no conclusions can be drawn about the localization of this residue in the enzyme. Ser^{367} is a conserved residue located in the adenine-binding part of the FAD-binding domain, and the S367A mutant exhibits indeed reduced FAD binding. However, this mutant has still some residual activity (4% of wild type). Probably, the overall tertiary structure is considerably disturbed by this mutation.

Clear differences are observed between the absorption spectra of purified MBP-alkyl-DHAP synthase recorded in either the presence or absence of SDS. In the absence of SDS significant aspecific light scattering is observed, which perturbs the absorption spectra. Because the buffer does not exhibit any absorbance above 300 nm, this must be due to the presence of aggregates. Possibly, as the FAD cofactor is fully reactive with substrate (Fig. 6), the apoform of alkyl-DHAP synthase present in the sample is less stable and tends to aggregate. Furthermore, two clear shoulders are observed aligning the absorbance maximum at 458 nm of the native enzyme, which disappear after SDS denaturation. Such shoulders are frequently observed in native flavoproteins (31, 32) and generally indicate that the isoalloxazine part of the FAD molecule is in an environment shielded from solvent.

Based on spectral properties, we could show that the flavin part of the cofactor is actively involved in the reaction mechanism (Fig. 5). Upon addition of the substrate palmitoyl-DHAP, the formed absorption spectrum of the enzyme indicates that the FAD cofactor is efficiently reduced. Apparently, palmitoyl-DHAP directly reacts with the flavin cofactor donating two electrons to the isoalloxazine ring. However, when long chain fatty alcohol (hexadecanol) is added to the mixture, the FAD cofactor is reoxidized.

Stopped flow measurements indicated a monophasic reduction of the FAD cofactor by palmitoyl-DHAP without any detectable intermediates (Fig. 6). The spectrum formed upon reaction with palmitoyl-DHAP represents a nearly fully reduced enzyme species, indicating that reduction is irreversible. Further, no evidence could be obtained supporting formation of a flavin N5 adduct during reduction because the reduced spectrum did not show any atypical features and was not fluorescent when excited at 360 nm (data not shown) (33). Therefore, we assume that two electrons are directly transferred from the substrate to the cofactor yielding $FADH_2$. The rate of reduction is in the same order as the turnover rate for the enzyme, whereas reoxidation by hexadecanol of the reduced species appears to be a relatively fast process. Combined with the observation that oxidized enzyme is detected during turnover (Fig. 5, *spectrum C*), these results suggest that the reductive half-reaction is a major factor in determining the turnover rate. These results clearly demonstrate the essential role of the FAD cofactor in alkyl-DHAP synthase catalysis. As in other oxidoreductases, the FAD cofactor in alkyl-DHAP synthase cycles between the oxidized state and reduced state. By unraveling this unexpected and remarkable functional role of the flavin cofactor in alkyl-DHAP synthase, we have broadened the range of reactions known to be catalyzed by flavoproteins.

Earlier studies on the mechanism of the alkyl-DHAP synthase reaction have shown that the pro-R hydrogen of the C-1 in the DHAP moiety is lost during the reaction (8–10) and that the enzyme can also exchange the fatty acyl group in acyl-DHAP for one from the medium (12). In both reactions the ester linkage in acyl-DHAP is cleaved by an alkyl oxygen fission, *i.e.* between the C-1 atom of the DHAP moiety and the oxygen of the acyl chain, in which both oxygens from the ester linkage are retained in the released fatty acid (6, 7). In crude microsomal systems the amount of the pro-R hydrogen lost exceeded the formation of alkyl-DHAP (8, 11), presumably because of the presence of free fatty acids either from the enzyme preparation or produced by hydrolysis of acyl-DHAP. However, with a delipidated and 1000-fold purified enzyme preparation devoid of acyl-DHAP acylhydrolase activity, the pro-R hydrogen loss became equivalent to the amount of the fatty acid exchange (8).

Thus, the interpretation of studies on the mechanism of alkyl-DHAP synthase has been seriously hampered in the past by the use of crude enzyme preparations exhibiting disturbing side reactions. However, the acyl exchange reaction appears to be a genuine property of alkyl-DHAP synthase that we have confirmed with the recombinant enzyme (14).

Based on the findings presented in this paper that alkyl-DHAP synthase contains FAD, which becomes reduced upon addition of acyl-DHAP, it is tempting to assume that the pro-R hydrogen is lost in a step in which the C-1 atom of the DHAP moiety becomes oxidized to yield an intermediate X of which the precise structure still has to be resolved. To investigate whether such an intermediate would be covalently linked to the enzyme, as was proposed in the reaction mechanism put forward previously by Brown and Snyder (8), the enzyme was incubated with palmitoyl-[^{14}C]DHAP and then analyzed by SDS-PAGE. No ^{14}C label was detected in the enzyme protein band supporting a noncovalent binding of the intermediate. When the incubation mixture of enzyme and palmitoyl-[^{14}C]DHAP was distributed over chloroform/water layers, a clear increase in water-soluble radioactivity was seen in comparison with similar extractions without enzyme. This indicates that the enzymatic reaction produces not only reduced cofactor but also a water-soluble intermediate that can be extracted from the enzyme. At present we cannot completely exclude the possibility that this water-soluble compound represents DHAP formed by hydrolysis of palmitoyl-DHAP rather than an intermediate formed in dependence of FAD. Yet the release of palmitic acid followed by reduction of the enzyme flavin by the DHAP is considered a less likely reaction sequence. First, this sequence would require an additional enzymatic activity, namely a separate acyl-ester hydrolysis reaction. Brown and Snyder (8) concluded from earlier studies on the reaction mechanism of this enzyme that although whole microsomes contain at least two acylhydrolases for acyl-DHAP, purified preparations of alkyl-DHAP synthase do not form DHAP from acyl-DHAP. Assuming this is true it means that no hydrolase activity *per se* is present in the enzyme. Second, acylhydrolase reactions are known to proceed by acyl oxygen fission involving cleavage of the acyl to oxygen bond (6, 34). A separate hydrolase step is therefore difficult to reconcile with the observations that both oxygens from the acyl ester bond are retained by the fatty acid (6, 7). Third, and more conclusive, we found that enzyme flavin is not reduced upon addition of dihydroxyacetonephosphate. This result was already expected from earlier observations. If the oxidized intermediate X would be formed from DHAP, either produced from acyl-DHAP in a separate deacylation step or from added DHAP, then this added DHAP would be expected to be a substrate for alkyl-DHAP synthesis. Early experiments by Hajra (35) have shown that acyl-DHAP, but not DHAP, is the substrate for alkyl-DHAP synthesis. Thus, the FAD-dependent formation of the oxidized DHAP moiety intermediate X directly from palmitoyl-DHAP is considered more plausible than its formation from DHAP.

In accord with and extending previous proposals (6, 8, 36), the oxidized DHAP moiety intermediate X in its binary complex with $FADH_2$-enzyme is then postulated to react with either a fatty alcohol or a fatty acid. At least the reaction with a fatty alcohol to generate the product alkyl-DHAP appears to be accompanied by regeneration of the FAD cofactor (Fig. 5). This postulate suggests that the enzyme may act via an ordered sequential mechanism, unless one considers the binary complex of reduced enzyme and oxidized DHAP moiety intermediate X as the modified enzyme intermediate obligatory in a ping-pong reaction in which the first product (fatty acid) is formed before the second cosubstrate (alcohol) comes in. This would be in line with the most recent kinetic analyses, which favored a ping-pong mechanism (13, 14). However, this interpretation was entirely based on the appearance of a set of parallel lines in double-reciprocal plots of reaction velocity *versus* palmitoyl-DHAP concentrations measured at different hexadecanol concentrations. In theory, an ordered sequential mechanism requires that the family of lines in such double-reciprocal plots intersect to the left of the ordinate. However, as pointed out by Dalziel (37) and Porter *et al.* (38), such intersections may be so far to the left that the lines will appear to be parallel, and the ordered sequential mechanism will behave like a ping-pong mechanism. A full analysis of all intermediates and true kinetic constants is required to decide between these reaction mechanisms.

In summary, this paper has shown the presence of the cofactor FAD in alkyl-DHAP synthase and documents its requirement for enzyme activity and its reduction and reoxidation upon stepwise addition of substrates. Stopped flow studies established that the reduction is considerably slower than the reoxidation. In addition, these experiments served to demonstrate that the reduction of flavin takes place in a single step, with no evidence for the formation of radical intermediates or charge-transfer complexes and that the reduced flavin is formed at rates sufficient to account for the catalytic activity of the enzyme. We believe these new findings set the stage for future detailed kinetic and mechanistic studies along completely new lines in comparison with the previous studies on the mechanism of the unique enzymatic reaction of glycerol-ether bond formation.

Acknowledgment—The use of the stopped flow instrument at the Laboratory of Biochemistry, Wageningen Agricultural University, is gratefully acknowledged.

REFERENCES

1. Hajra, A. K., and Bishop, J. E. (1982) *Ann. N. Y. Acad. Sci.* **386,** 170–182
2. Van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A., and Tager, J. M. (1992) *Annu. Rev. Biochem.* **61,** 157–197
3. Schrakamp, G., Roosenboom, C. F. P., Schutgens, R. B. H., Wanders, R. J. A., Heymans, H. S. A., Tager, J. M., and van den Bosch, H. (1985) *J. Lipid Res.* **26,** 867–873
4. De Vet, E. C. J. M., Ijlst, L., Oostheim, W., Wanders, R. J. A., and van den Bosch, H. (1998) *J. Biol. Chem.* **273,** 10296–10301
5. Wanders, R. J. A., Schutgens, R. B. H., and Barth, P. G. (1995) *J. Neuropathol. Exp. Neurol.* **54,** 726–739
6. Friedberg, S. J., Weintraub, S. T., Peterson, D., and Satsangi, N. (1987) *Biochem. Biophys. Res. Commun.* **145,** 1177–1184
7. Snyder, F., Rainey Jr., W. T., Blank, M. L., and Christie, W. H. (1970) *J. Biol. Chem.* **245,** 5853–5856
8. Brown, A. J., and Snyder, F. (1983) *J. Biol. Chem.* **258,** 4184–4189
9. Friedberg, S. J., and Heifetz, A. (1971) *Biochemistry* **14,** 570–574
10. Davis, P. A., and Hajra, A. K. (1979) *J. Biol. Chem.* **254,** 4760–4763
11. Friedberg, S. J., Gomillion, D. M., and Strotter, P. L. (1980) *J. Biol. Chem.* **255,** 1074–1079
12. Davis, P. A., and Hajra, A. K. (1981) *Arch. Biochem. Biophys.* **211,** 20–29
13. Brown, A. J., and Snyder, F. (1982) *J. Biol. Chem.* **257,** 8835–8839
14. De Vet, E. C. J. M., and van den Bosch, H. (1999) *Biochim. Biophys. Acta* **1436,** 299–306
15. De Vet, E. C. J. M., Zomer, A. W. M., Lahaut, G. J. H. T. J., and van den Bosch, H. (1997) *J. Biol. Chem.* **272,** 798–803
16. De Vet, E. C. J. M., van den Broek, B. T. E., and van den Bosch, H. (1997) *Biochim. Biophys. Acta* **1346,** 25–29
17. De Vet, E. C. J. M., Prinsen, H. C. M. T., and van den Bosch, H. (1998) *Biochem. Biophys. Res. Commun.* **242,** 277–281
18. De Vet, E. C. J. M., and van den Bosch, H. (1998) *Biochem. Biophys. Res. Commun.* **252,** 629–633
19. De Vet, E. C. J. M., Biermann, J., and van den Bosch, H. (1997) *Eur. J. Biochem.* **247,** 511–517
20. Zomer, A. W. M., de Weerd, W. F. C., Langeveld, J., and van den Bosch, H. (1993) *Biochim. Biophys. Acta* **1170,** 189–193
21. Thai, T-H., Heid, H., Rackwitz, H-R., Hunziker, A., Gorgas, K., and Just, W. W. (1997) *FEBS Lett.* **420,** 205–211
22. Biermann, J., Just, W. W., Wanders, R. J. A., and van den Bosch, H. (1999) *Eur. J. Biochem.* **261,** 492–499
23. Mattevi, A, Fraaije, M. W., Mozzarelli, A., Olivi, L, Coda, A, and van Berkel, W. J. H. (1997) *Structure* **5,** 907–920
24. Fraaije, M. W., van Berkel, W. J. H., Benen, J. A. E., Visser, J., and Mattevi, A. (1998) *Trends Biochem. Sci.* **23,** 206–207
25. Muh, U., Cinkaya, I., Albracht, S. P., and Buckel, W. (1996) *Biochemistry* **35,** 11710–11718
26. Macheroux, P., Bornemann, S., Ghisla, S., and Thorneley, R. N. (1996) *J. Biol.*

Chem. **271,** 25850–25858
27. Kellermann, O. K., and Ferenci, T. (1982) *Methods Enzymol.* **90,** 459–463
28. Kuichi, K. Nisikimi, M., and Yagi, K. (1982) *Biochemistry* **21,** 5076–5082
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
31. De Jong, E., van Berkel, W. J. H., van der Zwan, R. P., and de Bont, J. A. M. (1990) *Eur. J. Biochem.* **208,** 651–657
32. Ghisla, S., and Massey, V. (1980) *J. Biol. Chem.* **255,** 5688–5696
33. Fraaije, M. W., van den Heuvel, R. H. H., Roelofs, J. C. A. A., and van Berkel, W. J. H. (1998) *Eur. J. Biochem.* **253,** 712–719
34. Wells, M. A. (1971) *Biochim. Biophys. Acta* **248,** 80–86
35. Hajra, A. K. (1970) *Bichem. Biophys. Res. Commun.* **39,** 1037–1044
36. Hajra, A. K. (1995) *Prog. Lipid Res.* **34,** 343–364
37. Dalziel, K. (1962) *Biochem. J.* **84,** 244–254
38. Porter, D. J. T., Voet, J. G., and Bright, H. J. (1977) *J. Biol. Chem.* **252,** 4464–4473