The detoxification of formic acid by 10-formyl tetrahydrofolate dehydrogenase *in Apis mellifera*: capabilities and mechanism.

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I hereby declare that I have completed the submitted dissertation independently and without the use of sources and aids other than those indicated. I have marked as such all statements that are taken literally or in content from other writings. This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

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List of abbreviations

Abbreviation	Meaning
10-FTHFDH	10-formyl tetrahydrofolate dehydrogenase
6x-HisTag	6x-Histidine Tag
ATP	adenosine triphosphate
Ala	alanine
A673	alanine at position 673
A707	alanine at position 707
E673A	alanine exchange of cysteine at position 673
C707A	alanine exchange of cysteine at position 707
Å	Ångström
A. mellifera	*Apis mellifera*
Asp	aspartic acid
E673D	aspartic acid exchangeof glutamic acid at position 673
BLAST	Basic Local Alignment Search Tool
BV	bed volume
CO_2	carbon dioxide
C	carbon
cm	centimeter
R^2	coefficient of determination
cDNA	complementary deoxyribonucleic acid
CI	confidence intervall
C707	cysteine at position 707
Cys	cysteine
°C	degrees celsius
DNA	deoxyribonucleic acid
ddH2O	double destilled water
E	east
eGFP	enhanced green fluorescence protein
EDTA	ethylenediaminetetraacetic acid

EU	European Union
DE	Germany
Glu	glutamic acid
E673	glutamic acid at position 673
g	gram
× g	gravitational force (earth= 9.8m/s)
h	hour
HCl	hydrochloric acid
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	kilo dalton
kg	kilogram
LD_{50}	lethal dose 50
Vmax	limiting rate (Michaelis Menten)
l	liter
mRNA	messenger ribonucleic acid
Km	Michaelis-Menten constant
µmol	mikro mole
µg	mikrogram
mM	millimolar
mg	milligram
ml	milliliter
min	minute
M	molar
nm	nanometer
Ni-NTA	nickel nitrilotriacetic acid
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
N	north
NMR	nuclear magnetic resonance
C_1	one-Carbon
ORF	open reading frame

PABA	para-aminobenzoic acid
pFBD	pFastBacDual
F870	phenylalanine at position 808
PBS	phosphate buffered saline
PES	polyethersulfone
PCR	polymerase chain reaction
KOH	potassium hydroxide
pH	potentia hydrogenii
R. norvegicus	*Rattus norvegicus*
RNA	ribonucleic acid
RMSD	root-mean-square deviation of atomic position
rpm	rounds per minute
THF	tetrahydrofolate
THFDH	tetrahydrofolate dehydrogenase
tRNA	transporter RNA
Tris	tris(hydroxymethyl)aminomethane
USA	United States of America
U	units
V. destructor	*Varroa destructor*

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1. Summary:

Important ecological und economic functions are performed by different pollinators. *Apis mellifera* being one of the most important ones, as it is managed by beekeepers worldwide. In recent years, the obligate ectoparasite *Varroa destructor* grew of importance as the impact on honey bee hives grew more apparent, as research has shown that it not only feeds on haemolymph and fatbody and therefore weakens brood and adults directly, but also transmits different viral, bacterial and fungal diseases. A common treatment against *Varroa destructor* is formic acid fumigation. The upsides include high efficacy against *Varroa destructor* and no resistance or cross resistance development, as it is the case in synthetic treatment options such as amides (e.g. Amitraz). Additionally formic acid is certified for organic farms application. The biggest downsides of the treatment include *Apis mellifera* mortality through all clades, as well as a narrow therapeutic index, as the treatment is reliant on humidity, bee density, temperature and mode of application. Even though the treatment has been used for many years, very little is known about the detoxification mechanism in *Apis mellifera* and *Varroa destructor*.

Through recombinant expression of the 10-formyl-tetrahydrofolate dehydrogenase (10-FTHFDH), suspected to be involved in the detoxification of formic acid in *Apis mellifera*, the first study showed, that the enzyme is involved in the detoxification process, with similar activity known in mammalian counterparts. Additionally, as the sequence similarity of the insect 10-FTHFDH to the mammalian 10-FTHFDH is very low the second study focused on verifying the active residues and mode of action of the *Apis mellifera* enzyme. Through an interdisciplinary approach combining protein prediction, protein modelling and protein mutagenesis, it was verified, that the unsimilar proteins share the same active residues and function.

1. Zusammenfassung:

Wichtige ökologische und wirtschaftliche Funktionen werden von verschiedenen Bestäubern erfüllt. *Apis mellifera* ist einer der wichtigsten, da sie von Imkern weltweit gehalten wird. In den letzten Jahren hat der obligate Ektoparasit *Varroa destructor* an Bedeutung gewonnen, da die Auswirkungen auf das Überleben

der Bienen immer deutlicher wurden. Die Forschung hat gezeigt, dass sie sich nicht nur von Hämolymphe und Fettkörper ernähren und dadurch die Brut und die erwachsenen Bienen direct schwächt, sondern auch verschiedene virale, bakterielle und pilzliche Krankheiten überträgt. Eine gängige Behandlung gegen *Varroa destructor* ist die Behandlung durch Ameisensäureverdunstung. Zu den Vorteilen gehören eine hohe Wirksamkeit gegen *Varroa destructor* und keine Entwicklung von Resistenzen oder Kreuzresistenzen, wie dies bei synthetischen Behandlungsoptionen wie Amiden (z. B. Amitraz) der Fall ist. Außerdem ist Ameisensäure für die Anwendung in Biobetrieben zertifiziert. Zu den größten Nachteilen der Behandlung gehören die Sterblichkeit von *Apis mellifera* in allen Kasten sowie ein enger therapeutischer Index, da die Behandlung von der Luftfeuchtigkeit, der Bienendichte, der Temperatur und der Art der Anwendung abhängt. Obwohl die Behandlung seit vielen Jahren eingesetzt wird, ist nur sehr wenig über den Entgiftungsmechanismus bei *Apis mellifera* und *Varroa destructor* bekannt.

Durch rekombinante Expression der 10-Formyl-Tetrahydrofolat-Dehydrogenase (10-FTHFDH), von der vermutet wird, dass sie an der Entgiftung von Ameisensäure in *Apis mellifera* beteiligt ist, konnte in der ersten Studie bestätigt werden, dass das Enzym am Entgiftungsprozess beteiligt ist, ähnlich wie die bekannten Gegenstücke bei Säugetieren. Da die Ähnlichkeit des 10-FTHFDH der Insekten mit dem 10-FTHFDH der Säugetiere sehr gering ist, konzentrierte sich die zweite Studie auf die Verifizierung des aktiven Zentrums und der Wirkungsweise des *Apis mellifera*-Enzyms. Durch einen interdisziplinären Ansatz, der Proteinvorhersage, Proteinmodellierung und Proteinmutagenese kombiniert, konnte nachgewiesen werden, dass die unähnlichen Proteine die gleichen aktiven Reste und die gleiche Funktion haben.

2. Introduction

Our ecosystems provide us with essential resources like building materials, food and energy. The sun, being the ultimate source of energy in many ecosystems is used by plants and microorganisms to produce biomass through photosynthesis. These organisms absorb specific wavelengths (400-700nm) using the pigment chlorophyll and convert it to chemical energy by converting atmospheric CO_2 to carbon-containing energy sources like lipids, proteins and sugars (Chapin et al., 2002) . Plants as resources for direct consumption or feed production for animals are hugely reliant not only on the sun light as energy source, but on different pollinators as well, as 87% of all plants are reliant on pollination (Ollerton et al., 2011). Different classes of animals are involved in the pollination process, including lizards (Olesen & Valido, 2003), birds (Whelan et al., 2008), flying mammals (Cox et al., 1991), non-flying mammals (Carthew & Goldingay, 1997), and insects (Kevan & Baker, 1983). Of the different classes of pollinators, insects are considered to be the most important pollinators (Ollerton et al., 2011).

The on-going mass extinction of insects and biodiversity in general (Ceballos et al., 2015) bears great problems for the proper functioning of ecosystems. It is likely that declining numbers of pollinating mammals, birds (Regan et al., 2015) and insects (Schachat & Labandeira, 2021) will result in significant reduction in food production, as 70-80% of fruits and vegetables produced for human consumption are reliant on pollinators (Klein et al., 2007). Especially insects face pressure by different factors including intensive land use, climate change, invasive species and the spread of different pathogens (Potts et al., 2010). Hallmann et al. (2017) revealed in their 27-year long population observation study, that the insect biomass in several German protected areas declined by 76% which averages an approximately 3% loss per year. This loss of insects is not unique to Germany, as other parts of the world are severely hit by pollinator loss as well (Kluser & Peduzzi, 2007). Especially regions in China are in dire position. Here The pollination service is not conducted by insects any longer, but by manual labour by humans, because the insect population is not dense enough (Partap & Ya, 2012). If we fail to address this mayor problem of pollinator decline, we may face similar consequences worldwide, as in other regions, including Germany, the majority of pollination in agricultural context is performed by domesticated honey bees.

2.1. *Apis mellifera:* biology, importance and struggles

Apis mellifera, the western honey bee, is one of the most common and economically valued pollinators in agriculture. The natural habitat of *A. mellifera* ranges from Europe and the Middle East to Asia and Africa. Due to the wide distribution of the species, *A. mellifera* is classified into 4 lineages based on their morphological traits and genetic identity (Cornuet & Garnery, 1991; Franck et al., 2000; Ruttner et al., 1978). These characteristics span from body and wing morphology, over the identification of isoenzyme, to the identification of polymorphisms in mitochondrial DNA.

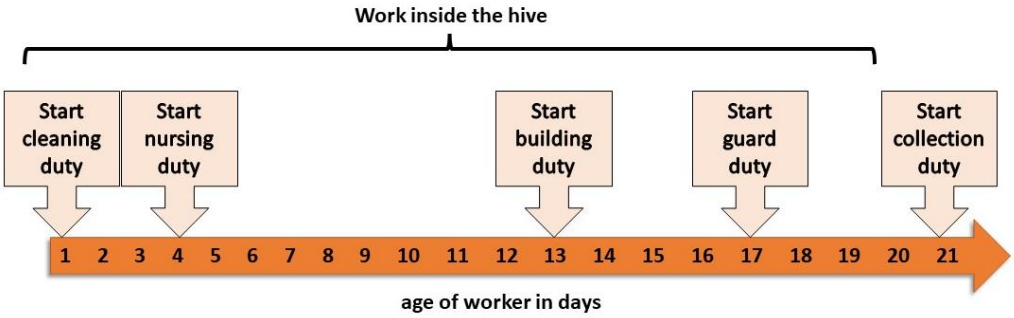

Figure 1 workdivision in honey bees. This figure shows the order in which the worker bees divide the labor depending on their age. Adapted from Pohl et al. 2017

As an eusocial insect *A. mellifera* lives in colonies with divided labour tasks (Pohl 2017). In addition to dividing labour, the colony consists of three different clades: Workers, drones and the queen. While the queen is responsible for egg laying and keeping the colony structure in line, the worker bees perform different tasks depending on their age (Figure 1). After emerging the worker bees start to clean out brood cells and the hive in general, after about 3 days the hypopharingal gland, which produces vital proteins for larvae development, is fully developed and the worker starts to feed larvae and capping the brood when ready for metamorphosis. After ten additional days the wax glands on the abdomen of the honey bees is in its prime and the construction of honey combs is performed. Adding four more days to the development allows for the active duty as the defence of the hive, as the poison bladder and sting are now fully potent. After a couple more days the honey bee starts to collect nectar, honey and propolis, until their dying day. Drones are solely important for sexual reproduction (Pohl, 2017).

Honey bees provide essential ecological and economical functions as the main plant pollinators for agriculture and horticulture (Havens & Vitt, 2016; Hung et al.,

2018). Many species consumed by humans are reliant on honey bee pollination (Paudel et al., 2015). In the United States, managed honey bee colonies declined from a peak in 1940 of around 6 million colonies to just over 2 million colonies in 2008 (Pettis & Delaplane, 2010). Insect problematics, such as climate change, pesticide usage, monocultures and prevalence and emergence of pathogens apply for honey bees as well, if not even more severe. In agricultural systems, honey bees are often exposed to different agrochemicals such as fertilizers, herbicides and insecticides. Fertilizers are not expected to have negative effects on honey bees (Siede et al., 2013), while herbicides (Farina et al., 2019; Motta et al., 2020) and insecticides (Belzunces et al., 2012; Halm et al., 2006; Lundin et al., 2015) reportedly impair learning, survival and orientation. The consequences of the impaired learning leads to higher rates at which honey bees provide false directions to newly found feeding sides. The problem with the reduction of orientation skills leads to an additional reduction in survivability of individuals, as they may not find their way back to the hive, as well as a reduction of survivability of the hive in general, as resources cannot be acquired as efficient as needed or if too many individuals do not find their way back home the colony might collapse.

Another challenge for honey bee health are pathogens. Within the last few decades honey bee viruses such as deformed wing virus, sacbrood virus and acute israeli bee paralysis virus, have grown to be more prevalent than ever (Brutscher et al., 2016; Chen & Siede, 2007). Viruses in honey bees are transmittable both horizontally and vertically, meaning that viruses may spread within a generation from individual to individual (horizontally), as well as from mother to offspring (vertically) (Chen et al., 2006). In addition to viruses, bacteria, for example *Paenibacillus larvae* (American foulbrood) and fungi, e.g. *Ascosphere apis* (chalkbrood), bear additional threats for the health of *A. mellifera* (Genersch, 2010; Heath, 1982). *P. larvae* is known to be one of the most deleterious diseases for the honey bee, as an infection with this pathogen often causes the collapse of the hive, as it rapidly affects larvae within the whole hive. The spores of the bacterium are taken up by cleaning bees which clean the dead larvae from their cells, they then distribute these spores to other larvae by feeding, additionally the spread of this pathogen and others is exacerbated by malpractices of beekeepers, which switch hive material between their hives without proper investigation of symptoms (Genersch, 2010; Rosenkranz et al., 2010; Stephan et al., 2020). Additionally Mill et al. (2014) showed that outbreaks of American

foulbrood build clusters of aggregation in a range of 10 to 30 km which prevail between one and up to five years.

2.2. Varroa destructor: biology, threat and treatment options

The most fatal problem, in the web of severe difficulties *A. mellifera* faces in the current world, is the ectoparasitic mite *Varroa destructor* (Anderson & Trueman, 2000), which feeds on haemolymph and the fat body of honey bee pupae and adults (Ramsey et al., 2019). In addition to directly weakening *A. mellifera*, *V.destructor* is known to play a key role in viral and bacterial pathogen transmission, including but not limited to deformed wing virus, sacbrood virus, acute israeli bee paralysis virus and *P. larvae* (De Rycke et al., 2002; Shen et al., 2005; Sumpter & Martin, 2004). The reproductive cycle of *V. destructor* is divided into two phases, the phoretic phase and reproductive phase. In the phoretic phase, adult female *V. destructor* are carried by adult bees throughout the hive, in which the mites may attach to other honey bees. In the reproductive phase, mites detach themselves from bees inside a brood cell shortly before the capping of the brood cell. After capping the female lays 5-6 eggs where the first egg is unfertilized and develops into male offspring. This male offspring will proceed to sexually reproduce with the female mites which hatch from fertilized eggs (Figure 2) (Rosenkranz et al., 2010). This phase where *V. destructor* is trapped within the capped brood cell is often used to apply a biomechanical treatment. Biomechanical treatments function due to the fact that *V. destructor* heavily prefers drone brood cells to reproduce (Fuchs, 1990). In this case, the capped drone brood is removed from the hive and a huge part of the *V. destructor* population is eliminated.

In addition to biomechanical treatment options, other chemical treatments have proven successful. Previously preferred treatments include synthetic chemicals such as pyrethroids and amidines (Le Conte et al., 2010; Mitton et al., 2016), which are applied by either strips, sprays or fumigation. The main problem with such chemicals involves the development of resistances and cross-resistances (Le Conte et al., 2010; Mitton et al., 2016; Thompson et al., 2002). Another approach is the application of essential oils, which work against different mites (Gregorc & Planinc, 2005), but have negative impacts on bee survival and behaviour (Gashout & Guzmán-Novoa, 2009; Mondet et al., 2011), as well as unwanted contamination of bee products (Bogdanov et al., 1998). Other treatment options include organic acids such as oxalic acid, lactic acid and formic acid. Whereas oxalic acid is only used during the winter, as it has brood

damaging properties (Rademacher et al., 2017), where the mode of action has not yet been investigated. Lactic acid on the other hand does not work in capped brood, as it is unable to penetrate the bees wax, and therefore is mainly used on artificially created swarms (Kraus & Berg, 1994).

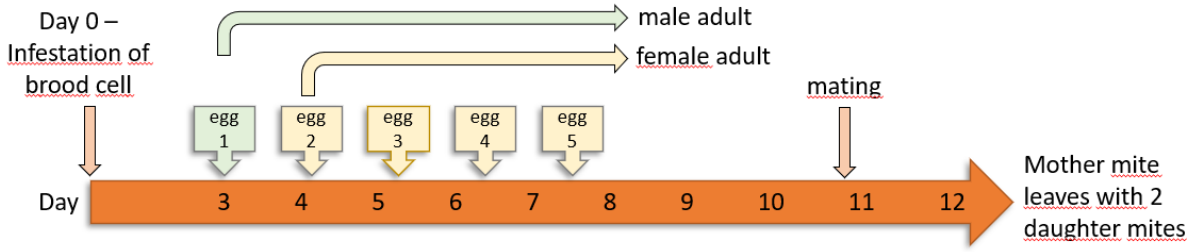

Figure 2 Reproductive cycle of *Varroa destructor* in honey bee brood cells. A female mite enters the uncapped brood cell shortly before capping. About three days after capping the first unfertilized egg is laid which will develop into a male. Thereafter four to five fertilized eggs are laid which will develop into females. Approximately 11 days after capping the siblings will mate and 2 daughter mites and the mother will leave the cell with their mother. Adapted from Rosenkranz et al. (2010).

2.3. Formic acid: An overview of use and detoxification

Formic acid is commonly used around the globe as a treatment for different mite species (Avila-Ramos et al., 2010; Girişgin & Aydin, 2010; Gregorc & Poklukar, 2003; Imdorf et al., 1999; Kraus & Berg, 1994). Even in nature birds are often observed to practice “anting”, where birds land in an ant hive to get the ants to attack them with their formic acid and distribute it in their feather coat, which is expected to be a natural way of formic acid treatment against mites (Morozov, 2015). The positive aspects of the applied treatment for honey bees include greater efficiency, usability on capped brood and a lack of resistance development in *V. destructor*. In contrast, negative aspects of formic acid use include the inability of treatment during harvest periods, as formic acid can penetrate capped cells, the strong dependence of efficiency on environmental factors such as humidity, hive strength and applicator used, as well as worker and queen mortality (Imdorf et al., 1999; Rosenkranz et al., 2010). Besides these negative impact the efficiency of the treatment outways those points, as a about 65% up to 80% of the *V. destructor* population is eliminated with correct application (Steube et al., 2021).

Little is known about the molecular mode of action of formic acid in honey bees, and especially how this organic acid is detoxified. Previous to this doctoral thesis one paper has been published which describes the dysregulation of genes in *Apis mellifera*

after exposure to formic acid (Genath et al., 2020). The most promising candidate gene is of the enzyme 10-formyl tetrahydrofolate dehydrogenase (10-FTHFDH; EC 1.5.1.6), being upregulated after exposure to fumigation treatment with formic acid. This enzyme is involved in the formate oxidation, for example in rats the formate oxidation is higher due to the higher amount of hepatic 10-formyl-THF and the higher activity of 10-FTHFDH (Johlin et al., 1989). The detoxification pathway involving this enzyme is divided into two steps: The first step involves the conversion of tetrahydrofolate (THF) to 10-formyl tetrahydrofolate (10-formyl-THF) by the enzyme 10-formyltetrahydrofolate synthase (Jaenicke & Brode, 1961), here the formic acid in the form of an formyl group is attached to the THF. Thereafter the aforementioned 10-FTHFDH catalyses the $NADP^+$ dependent reaction of 10-formyl THF to CO_2 and THF (Anguera et al., 2006; Johlin et al., 1989). It was hypothesized that the lower LD_{50} and thus higher toxicity in *V. destructor* compared to *A. mellifera* could be explained by the difference in morphology and body size. Because the surface area is much larger compared to the body mass in *V. destructor*, more formic acid would be absorbed through the body surface, as well as the lower buffering capabilities of *V. destructor* (Bolli et al., 1993). To date, neither physiological nor biochemical or molecular studies have been performed to explain the higher toxicity of formic acid on *V. destructor* compared to *A mellifera*. Additionally, the mechanism of formic acid detoxification in *V. destructor* appears to be different to *A. mellifera* as Genath et al. (2020) found an enzyme of a different family, flavin-containing mono oxygenase 5-like, to be upregulated after formic acid exposure. This difference between species may open the opportunity to optimize pathogen treatment with formic acid by inhibiting the *V. destructor* enzyme and supporting the involved enzymes in *Apis mellifera* by supplement feeding. For this reason, the basis of the mechanisms needs to be further investigated.

It is hypothesized that the 10- formyl tetrahydrofolate dehydrogenase of *A. mellifera* plays a vital role in the detoxification of formic acid, a commonly used treatment against Varroa destructor. To verify the functionality the enzyme is recombinantly expressed and tested in vitro, thereafter the molecular mechanism within this specific enzyme is analysed.

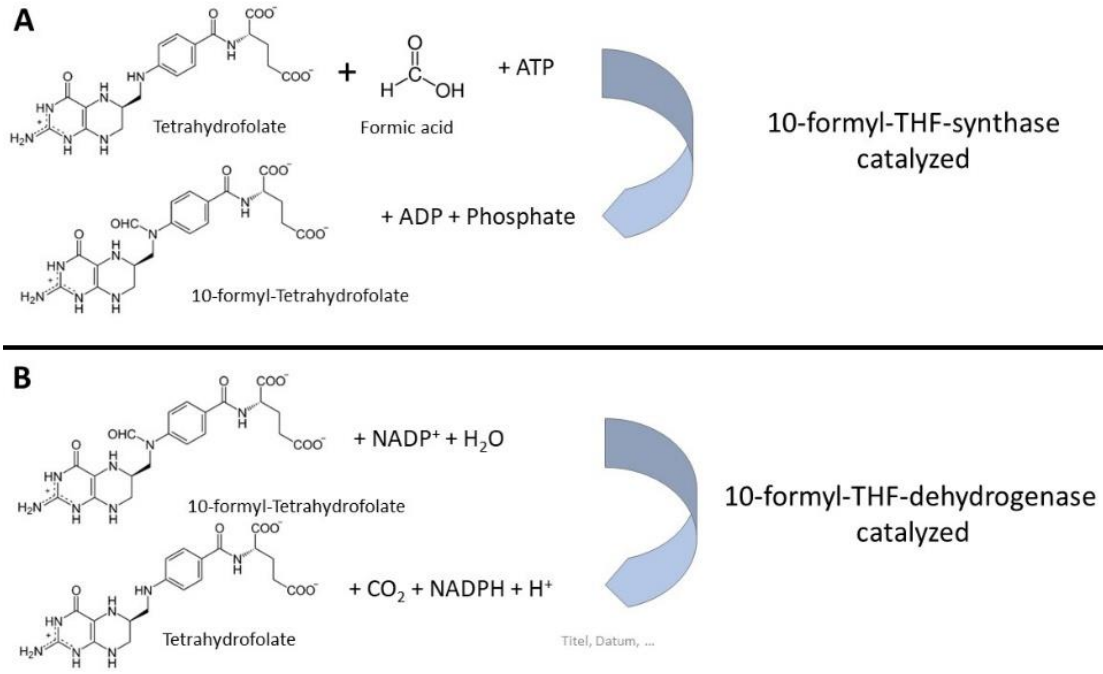

Figure 3 Reaction overview of the detoxification of formic acid in mammals. A) shows the first step reaction involving the 10-FTHF-synthase. B) shows the second part reaction involving the 10-FTHFDH

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A Detoxification Enzyme for *Apis mellifera* Newly Characterized by Recombinant Expression: 10-Formyl Tetrahydrofolate Dehydrogenase

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Honeybees are important managed pollinators that perform important ecological and economic functions. In recent decades, the obligate ectoparasite *Varroa destructor* severely affected survival of honeybees as it either feeds on hemolymph and fat bodies or acts as a vector for viruses. A common treatment against the varroa mite is formic acid, which has been used for many years by beekeepers. This treatment is known to be effective, but the therapeutic index is very narrow. Many beekeepers report negative effects of formic acid on bees, which include damage to brood, worker bee mortality, and queen loss. Little is yet known about the molecular mechanisms of formic acid detoxification in honeybees. Our previous study shows the upregulation of predicted 10-formyl tetrahydrofolate dehydrogenase (10-FTHFDH) transcripts in honeybees exposed to formic acid. Here, the predicted honeybee-specific 10-FTHFDH is recombinantly expressed, and its hydrolase and dehydrogenase activities are investigated. As a result, the enzyme shows similar dehydrogenase activity in comparison to known 10-FTHFDHs. This study provides further knowledge to better understand the detoxification mechanisms of formic acid in *Apis mellifera.*

Keywords: *Apis mellifera*, *Varroa destructor*, formic acid, detoxification, 10-FTHFDH, apiculture, honey bee (*Apis mellifera* L.)

IINTRODUCTION

Honeybees provide essential ecological and economic functions in our modern society. Honeybees are main pollinators for many agricultural and flowering plants in general (1, 2). Many plants are vastly reliant on pollination through bees, such as almonds, avocados, blueberries, onions, and many more (3). Overall, the economic value worldwide is estimated at up to 190 billion euros (4). For the last decades, a strong decline in honeybee populations is reported around the world. In the United States managed honeybee colonies declined from a peak in 1940 of around 6 million colonies to just over 2 million colonies in 2008 (5). This decline has remained constant over the last 10 years with an annual colony loss of about 40% (6). It is, therefore, predicted that one day the critical number may be reached at which there will no longer be enough bees and other pollinators
(7). In addition to malpractices of beekeepers, increased use of pesticides by farmers, and the emergence and prevalence of pathogens, varroa mites are one of the major factors in the loss of colonies (8, 9). *Varroa destructor* (10) as an obligate ectoparasitic mite that feeds on the fat body and hemolymph of larvae and adult bees (11) directly weakens the larvae and imagos. In addition,

varroa is known as a vector of various pathogens, including viruses, such as Deformed wing virus, Sacbrood virus, and Acute bee paralysis virus (12, 13) as well as bacterial pathogens like American foulbrood (14).

Early treatments against *V. destructor* are essential and comprise the usage of synthetic pyrethroids, such as Fluvalinate and amidines, such as Amitraz (8, 15). A common problem is the quick development of resistance, which also leads to cross- resistances against other pyrethroids (8, 16, 17). Additionally, due to the lipophilic character of the pyrethroids, residues of the chemicals can be found in bee products, such as wax and honey (18, 19). Alternatives to those synthetic chemical compounds are organic acids. Naturally occurring acids, such as oxalic, lactic, and formic acid, are licensed for application in varroa-infested hives in the EU, United States, and Canada as well as most of Latin America, including Argentina, Colombia, Costa Rica, and more (20–26). Formic acid has a very low risk of leaving residues in bee products compared with synthetic acaricides when used correctly (27, 28). The application of formic acid does not exclusively provide health benefits for honeybees. Many different factors influence the efficiency of the treatment, such as temperature, humidity, colony strength, and presence of larvae as well as type and position of used applicator (9, 21). Additionally, the therapeutic index is very narrow, which could lead to damaged larvae and juveniles. Even though formic acid has been used for many years to control varroa, the molecular mechanisms for detoxification are widely unknown in honeybees. Our recent data show that the mRNA of the enzyme cytosolic 10-formyl-tetrahydrofolate dehydrogenase is upregulated in honeybees treated with formic acid (29).

Tetrahydrofolate (THF) is an essential molecule involved in the universal one-carbon (C_1) metabolism, including purine and thymidine synthesis and homocysteine remethylation. The term "folate" in general includes molecules with three chemical parts: a pteridine ring, a para-aminobenzoic acid (PABA), and a polyglutamate tail. The bioactive form of folate is called tetrahydrofolate (THF), which is the reduced form of folic acid. The formyl group can exist in three different carbon oxidation states, all of which have different biochemical functions: 5,10-methylene-THF, 5-methyl-THF, and 10-formyl-THF (30). 10-formyl-THF is the most oxidized naturally occurring folate carbon. It is required for the *de novo* synthesis of purines. In proliferating cells *in vitro*, purine synthesis is the largest demand for 1C units (30, 31). In bacteria as well as in mitochondria, initiator methionine tRNAs are formylated by a process using 10-formyl-THF (32). The most remarkable property of 10-formyl-THF for our studies is that the C_1 unit can be completely oxidized to CO_2 in an $NADP^+$-dependent reaction, which could easily remove, for example, formic acid from the organism (33). As reported for mammals, the folate-dependent One-Carbon- Pool (C_1) is the most important detoxification pathway of formic acid, catalyzing the conversion of tetrahydrofolate (THF) to 10-formyltetrahydrofolate (10-THF) by a 10-formyltetrahydrofolate synthase (34). Subsequently, the aforementioned 10-formyltetrahydrofolate dehydrogenase catalyzes the $NADP^+$-dependent reaction of 10-THF to CO_2 and THF (35, 36). Formic acid is assumed to be toxic due to the inhibitory effect on the mitochondrial cytochrome oxidase, therefore, causing histotoxic hypoxia and acidosis (37, 38). The toxicity to mammals is highest after inhalation (LD_{50} of 7.4mg/l/4h in rats), but only low-to-moderate toxicity is observed with 145 mg/kg intravenous application in mice. No significant impacts on reproductivity, carcinogenicity, and genotoxicity have been found so far (39) with an exception in an *in vitro* study comparing the developmental toxicity on mouse and rat embryos after exposure to formic acid, where several defects, including open anterior and posterior neuropore were reported (40). These severe negative effects could not be confirmed *in vivo*, where the application of formic acid over several generations in rats does not result in negative effects (39). With LD_{50} ratios of 267 µg/ml/48 h for *A. mellifera* and 9µg/ml/48 h for *V. destructor*, the difference in tolerance between these two species is obvious (41). It was hypothesized that the lower LD50 and, thus, higher toxicity in *V. destructor* compared to *A. mellifera* could be explained by the difference in morphology and body size. Because the surface area is much larger compared to the body mass in varroa, more formic acid would be absorbed through the body surface. Apart from this hypothesis, neither physiological nor biochemical and molecular studies have been performed so far to explain the higher toxicity (42).

The aim of this study is to demonstrate and characterize the predicted function of the recombinantly expressed enzyme 10-formyl-tetrahydrofolate dehydrogenase of *Apis mellifera*. We show that this newly found insect enzyme has similar activities to previously described mammalian enzymes and, therefore, may play a key role in the detoxification of formic acid in honeybees.

MATERIALS AND METHODS

Apis mellifera Sampling

One-day-old worker bees were collected from the apiary of the Institute of Veterinary Biochemistry, Freie Universität Berlin, Berlin (52.42898 °N, 13.23762 °E) using one queen-right colony with *A. mellifera (carnica)* in the summer season 2020. Colonies were healthy, had enough food supply, and showed no symptoms of diseases or increased parasitism. Individuals were shock- frozen in liquid nitrogen and stored at −80°C until further use.

RNA Extraction

RNA extraction was performed using the Quick-RNA™ Miniprep Kit (Zymo Research Europe GmbH, Freiburg, DE). Briefly, individuals were lysed in a lysing Matrix S (MP Biomedicals, Heidelberg, DE) containing 1 ml of lysis buffer using a BeadBlaster (Benchmark Scientific, Edison, USA). Tubes were then centrifuged at 12,000 × g at 4°C for 10 min. Supernatant was transferred into a clean microcentrifuge tube containing 1.5x volume 100% ethanol. The solution was then used according to manufacturer's protocol. RNA was eluted in a total volume of 40 µl ddH_2O. Quantity and quality of total RNA was analyzed using an agilent RNA 6000 nano chip on a 2100 Bioanalyzer (Agilent Technologies, California,USA). Isolated RNA was stored at −80°C until use.

First Strand cDNA-Synthesis

ProtoscriptⓇII Transcriptase (New England Biolabs, Inc., Ipswich, USA) was used according to manufacturer's protocol.

Briefly, 1 µg DNA-free RNA was incubated with 1 µl d(T)23VN- Primer (50 µM) and 1 µl Random Primer Mix (50 µM) at 65◦C for 5 min in a total volume of 8 µl. Thereafter, 12 µl of Protoscript Mastermix was added, and the sample was incubated at 42◦C for 60 min and heat inactivated at 80◦C for 5 min. cDNA was then diluted by addition of 80 µl ddH2O and stored at −20◦C in adequate aliquots. To create a broad library, 5 µl of each sample was added to one microcentrifuge tube before freezing.

Sequence Alignment and BlastX

Clustal Omega (43) was used for sequence alignment. *Homo sapiens*, *Rattus norvegicus*, *Pongo abllei*, and *Mus musculus* amino acid sequences were retrieved from uniprot.org (Accesion Nr: O75891, P28037, Q5RFM9, Q8R0Y6), and predicted amino acid sequences of *A. mellifera* was retrieved from NCBI (Accesion Nr.: XP_026298140.1). A protein similarity summary was generated based on the DNA sequence of *A. mellifera* THFDH using BlastX (NIH).

Creation of pFBD-eGFP-Amel_10-FTHFDH Expression Vector

The open reading frame of the *A. mellifera* cytosolic 10-formyltetrahydrofolate dehydrogenase (Accession: XM_026442355.1) (Amel_10-FTHFDH) was amplified by polymerase chain reaction (PCR) using the primers Amel_FTHFDH_ORF_F/R (5′-ATGGCGCAACTCAAAGTGGC; 5′-CTAATATTCTACAGTGATAGTTTTTG). The PCR product was then subcloned into pJet1.2 vector (Thermo Scientific, Karlsruhe, DE) for sequencing and creation of a template for further use. The ORF-containing vector was used to create overhangs containing restriction sites (BamHI, NotI), and a 6x-HisTag at the N-terminus for later purification of the protein. pFastBacDual (pFBD) vector of the Bac-to-Bac System (Thermo Scientific, Karlsruhe, DE) with an enhanced green fluorescent protein (eGFP) cloned at the p10-promoter site was used as expression vector. The insert was created by PCR using the Amel_FTHFDH_BHI_HT_F and Amel_FTHFDH_NotI_R primers (5′-TCATACGGATCCATGCACCACCACCACCACCA CGCGCAACTCAAAGTGGC; 5′-TCATACGCGGCCGCCT AATATTCTACAGTGATAGTTTTTG). pFBD-eGFP was digested with appropriate restriction enzymes, and the vector was dephosphorylated using an Antarctic Phosphatase (New England Biolabs, Inc., Ipswich, USA) to prevent relegation. PCR product was ligated with the vector using a T4-ligase (New England Biolabs, Inc., Ipswich, USA) using standard protocols.

Creation of the Recombinant Bacmid

To create the recombinant Bacmid, Gibco™ Max Efficiency™ DH10Bac competent Cells (Thermo Scientific, Karlsruhe, DE) were transformed using 1 µg of pFBD-construct. Cells were thawed on ice, and 1 µg construct was added. The mixture was incubated for 30 min on ice, heat-shocked for 45 s at 42◦C, and transferred back to ice for 2 min. Then, 900 µl S.O.C medium were added. Culture was incubated for 4 h at in a shaking incubator at 225 rpm. Cells were plated on LB- medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 500 µg/ml X-Gal, and 1 µM IPTG. Plates were incubated for 48 h at 37◦C. White colonies were restreaked and Bacmid isolated using manufacturer's protocol.

Creation of Baculovirus

To create the recombinant baculovirus, Sf21 insect cells were transfected with 1 µg Bacmid DNA, and 6 µl Gibco™ Cellfectin™ II reagent (Thermo Scientific, Karlsruhe, DE) was used as suggested by the manufacturer. Successful transfection was monitored by expression of eGFP under an inverse fluorescent microscope DMI 6000B (Leica), and photos were taken using a DFC 365FX (Leica) camera. Virus stock was extracted by detaching cells from the flask and centrifuging at 3,000 × g for 5 min. Virus-containing supernatant was transferred into a sterile 15 ml centrifuge tube and stored safe from light at 4◦C until further use.

Expression and Purification of Recombinant Protein

To produce recombinant protein, Hi5 cells at 80–90% confluency were used, and 3×10^6 cells were seeded into a T175 flask (Sarstedt) containing 50 ml of Gibco™ ExpressFive™ SFM (Thermo). Next, 30 µl/ml virus stock was added, and cells were incubated at 27◦C for 4 days or until most cells showed eGFP expression. Cells were pelleted at 5,000 × g for 20 min at 4◦C. Pellets were resuspended in 20 ml PBS containing EDTA- free proteinase inhibitor cocktail (SIGMA). The suspension was sonified on ice using a sonifier 250 (Branson) for 4 min with an amplitude of 2 and at 20% energy. The suspension was cleared by centrifugation at 5,000 × g for 20 min at 4◦C. Protein- containing supernatant as well as PBS containing Imidazole at different concentrations [10 mM (Equilibration Buffer), 25 mM (Wash Buffer), 100, 150, 200, and 500 mM (Elution Buffer)] were particle-free filtered (0.4 µM pore size, PES); 2 ml bed-volume (BV) of HisPur™ Ni-NTA Resin (Thermo) was equilibrated with 5 BV Equilibration buffer. Protein was equilibrated with 20 ml equilibration buffer and added to the column. The column was washed with 20 BV wash buffer, and thereafter four elution fractions were obtained using four different concentrations of imidazole (100, 150, 200, and 500 mM). The whole purification was performed at 4◦C. Thereafter, to remove impurities and Imidazol from the enzyme, protein concentrators with a molecular weight cutoff of 50 kDa (Pierce) were used as suggested by the manufacturer.

Synthesis of 10-Formyl Tetrahydrofolate

To synthesize the substrate 10-formyl tetrahydrofolate, an established protocol by Rabinowitz et al. (44) was used. Briefly, 100 mg of dl-5-formyltetrahydrofolic acid (SIGMA) were dissolved in 8 ml of 1 M β-mercapto-ethanol (Roth). The pH was adjusted to 1.5 with HCl. The mixture was stored at 4◦C for at least 12 h. The solution, now containing dl-5,10-methenyltetrahydrofolic acid as a precipitate (bright yellow tint), was adjusted to a pH of 8 with KOH, purged with Argon, and incubated overnight at 4◦C in an evacuated vessel. The

TABLE 1 | Percentage identiy matrix, showing overall percentage identity between total 10-FTHFDH (T), Hydrolase domain of 10-FTHFDH (H), and dehydrogenase domain of 10-FTHFDH (D).

		Apis mellifera	Homo sapiens	Pongo abelii	Rattus norvegicus	Mus musculus
	T	100				
Apis mellifera	H	100				
	D	100				
	T	59.71	100			
Homo sapiens	H	55.39	100			
	D	69.65	100			
	T	59.71	98.34	100		
Pongo abelii	H	55.02	98.52	100		
	D	69.85	94.19	100		
	T	60.04	91.80	92.02	100	
Rattus norvegicus	H	56.51	93.70	93.70	100	
	D	69.85	92.95	93.36	100	
	T	60.04	92.35	92.68	97.67	100
Mus musculus	H	56.13	93.70	93.70	97.78	100
	D	70.06	94.19	94.61	98.34	100

Within mammals, the percentage identity is above 91% in all categories, whereas the percentage identity between total A. mellifera 10-FTHFDH and mammals is at about 60% with an increase in percentage identity in the dehydrogenase domain and a decrease in the hydrolase domain, with around 70 and 55%, respectively.

solution now containing 10-formyl tetrahydrofolate (clear color) was directly used for assays.

Enzyme Activity Assays

All assays were performed in a ClarioStar plus multimode-plate reader (BMG labtech); 100 mM β-mercapto-ethanol, 200 μM $NADP^+$ and 10 μg of purified enzyme were added to each well and incubated at 30°C for 2 min. Substrate was injected using built-in injectors at different concentrations. NADPH production was monitored at 340 nm for a period of 30 min. All substances were diluted in Tris/HCl buffer (pH 6.8–8.4) to a total of 100 μl. Km and V_{max} have been calculated using a molar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADPH. For the hydrolase activity assay, the abovementioned conditions were used, but $NADP^+$ was omitted and production of the product was monitored at a wavelength of 300 nm.

Analysis of Kinetic Data

Initial reaction rates were used to determine the respective enzyme activities. Kinetic parameters were derived by using GraphPad Prism version 9.0.2 (for Windows 10, GraphPad Software, San Diego, California USA, www.graphpad.com), which determines kinetic parameters from the Michaelis–Menten equation by using non-linear regression.

RESULTS

Sequence Alignment and BlastX

General sequence alignment of known 10-formyl tetrahydrofolate dehydrogenase (10-FTHFDH) sequences of *Homo sapiens*, *Rattus norvegicus*, *Pongo abelii,* and *Mus musculus* with the sequence of the *A. mellifera* 10-FTHFDH show a high percentage identity between mammals (ca. 91% homology). In contrast, the percentage homology between mammals and *A. mellifera* was at about 60% (**Table 1**). This value increased when only the specific sequence regions of the dehydrogenase domains were aligned (∼70% homology). When only the hydrolase domains were aligned, homology values decreased to 55%. BlastX revealed that the dehydrogenase domain is recognized as such, but the hydrolase domain is no longer recognizable by the analysis (**Figure 1A**). Sequence alignment also revealed that important regions such as E673 and C707 of the dehydrogenase domain, known to be key residues in the active site, are highly conserved (**Figure 1B**).

Amel_10-FTFDH Expression and Purification

10-formyl-tetrahydrofolate dehydrogenase of *A. mellifera* was successfully expressed and purified, containing a N-terminal 6x-HisTag, and 7×10^7 Hi5-Insect cells resulted in a total yield of ∼2 mg purified protein. Instead of using classical viral plaque assays, the expression of eGFP was used as a measure for a successful target protein production (**Figures 2A, B**). The protein identification by means of SDS-PAGE revealed successful expression and purification of 10-FTFDH (**Figure 2C**). The calculated size of the predicted protein is expected to appear at 100 kDa, which was verified by the gel. The non-specific weak band at 52 kDa could probably be a degradation product.

Enzymatic Activity and pH Dependence

We show that the expressed 10-formyl tetrahydrofolate dehydrogenase of *A. mellifera* shows dehydrogenase activity but does not show any hydrolase activity (SI1). The dehydrogenase activity was measured by monitoring the increase of absorbance

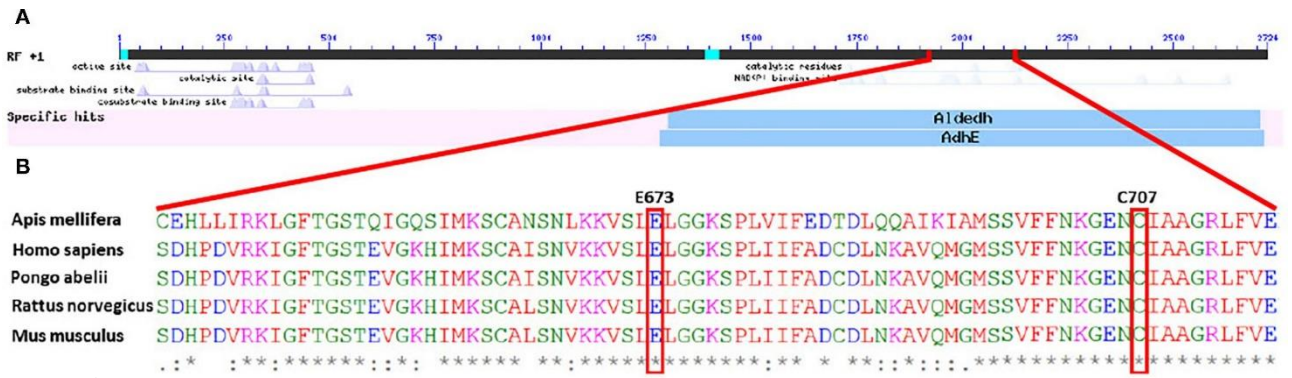

FIGURE 1 | (A) Graphic overview of BlastX of the DNA sequence of Amel_FTHFDH, showing specific hits for known enzymes. The dehydrogenase subunit is identified as such, whereas the potential hydrolase subunit is not identified. (B) Partial amino acid sequence alignment of different 10-formyl tetrahydrofolatedehydrogenases. Highlighted amino acids are important residues in the active sites of mammalian dehydrogenases.

at 340 nm, the maximum extinction peak of NADPH. Typical Michaelis–Menten kinetics are observed at all tested pH values (**Figure 3A**). The affinity of the enzyme for its substrate is indicated by its Km value. As depicted, the Km value is dependent on the pH with its optimum, indicated by low Km value, at pH 6.8 with a value of 2.455 µM. At pH 7.6 and 8.4, the Km increases to 9.596 µM and 2.961 µM, respectively (**Figure 3B**). The enzyme shows an optimal activity, which is indicated by a high Vmax, at pH 7.6 with a value of 0.4253 nM min^{-1}. At the pH of 6.8 and 8.4, the enzyme expresses an activity of 0.2907 nM min^{-1} and 0.1846 nM min^{-1}, respectively (**Figure 3C**).

DISCUSSION

This study shows for the first time that the predicted 10-formyl tetrahydrofolate dehydrogenase (10-FTHFDH) of *A. mellifera* (XM_026442355.1) can be expressed *in vitro,* and the resulting enzyme exhibits the expected dehydrogenase activity for the substrate 10-formyl tetrahydrofolate. In insects, there are to date no publications on the formic acid detoxifying enzyme 10- FTHFDH; in contrast, mammalian 10-FTHFDHs have been well- studied, particularly those of rat, mouse, and human (45–48).

Our study demonstrates an optimum of enzymatic activity at a neutral to basic pH. Similar results were previously reported for a recombinantly expressed 10-FTHFDH of *Rattus norvegicus* (49). The study reports the V_{max} value of about 0.095 µmol min^{-1} mg^{-1}. In comparison, using the same units, we report a V_{max} of 0.043 µmol min^{-1} mg^{-1} of enzyme. The K_m value usually depends on the pH at which the reaction takes place. In our case it has a maximum at a pH of 7.6. The Km value in our study,

9.6 µM, is in a similar range to that of rat, 5.5 µM, and pig, 7.5 µM (49, 50). In both studies, the maximum activity was at neutral to basic pH (7.6–7.7), which supports our findings. Thus, the activity of the enzyme 10-FTHFDH, characterized for the first time in an insect, is comparable to the known activity values for the previously described representatives from mammals.

All 10-FTHFDH described so far are divided into two domains, comprising (I) a hydrolase domain, which catalyzes the $NADP^+$-independent reaction of 10-formyltetrahydrofolate to tetrahydrofolate and formate and (II) a dehydrogenase domain, which catalyzes the $NADP^+$-dependent reaction of 10-formyl tetrahydrofolate to CO_2, tetrahydrofolate and water (45, 49). Tsybovsky and Krupenko (51) propose the following mechanism for the dehydrogenase catalysis. Glutamate E673 is hydrogen bonded to cysteine C707. The binding of NADP+ results in the rotation of the glutamate sidechain away from the cysteine, which simultaneously loses a proton; thereafter, the negatively charged sulfur of the cysteine forms a transient covalent bond with the C4 atom of the nicotinamide ring of the coenzyme. In the two phases of the dehydrogenase catalysis, acetylation and deacetylation, the cysteine functions as a catalytic nucleophile, whereas the glutamate is postulated to activate a water molecule in the deacetylation step. With the proposed mechanism, the two mentioned residues are of great importance. The amino acid sequence alignment showed a high percentage identity within the group of mammals analyzed, whereas the comparison of our honeybee 10-FTHFDH protein to the mammal enzymes shows a way lower percentage identity of about 60%. However, looking more closely at the specific domains, the dehydrogenase domain shows a marked increase in amino acid sequence homologies (from 60% to about 70%). Especially the previously mentioned functionally important residues and the regions in the surrounding area are highly conserved (**Figure 1B**). However, with an overall percentage identity of 70%, further studies should be performed to verify the active site. The hydrolase domain, on the other hand, has a lower percentage protein homology (about 55%), which could explain the loss of hydrolase function.

Formic acid toxicity is directly related to a burst of reactive oxygen species and oxidative damage in cells induced by formic acid (52). In contrast, folate plays an important role in reducing this oxidative stress (53), which would likely be explained by an increase in detoxification capacity. In humans, folate coenzymes are known to play a vital role in cellular homeostasis. Animals in general cannot synthesize folate *de*

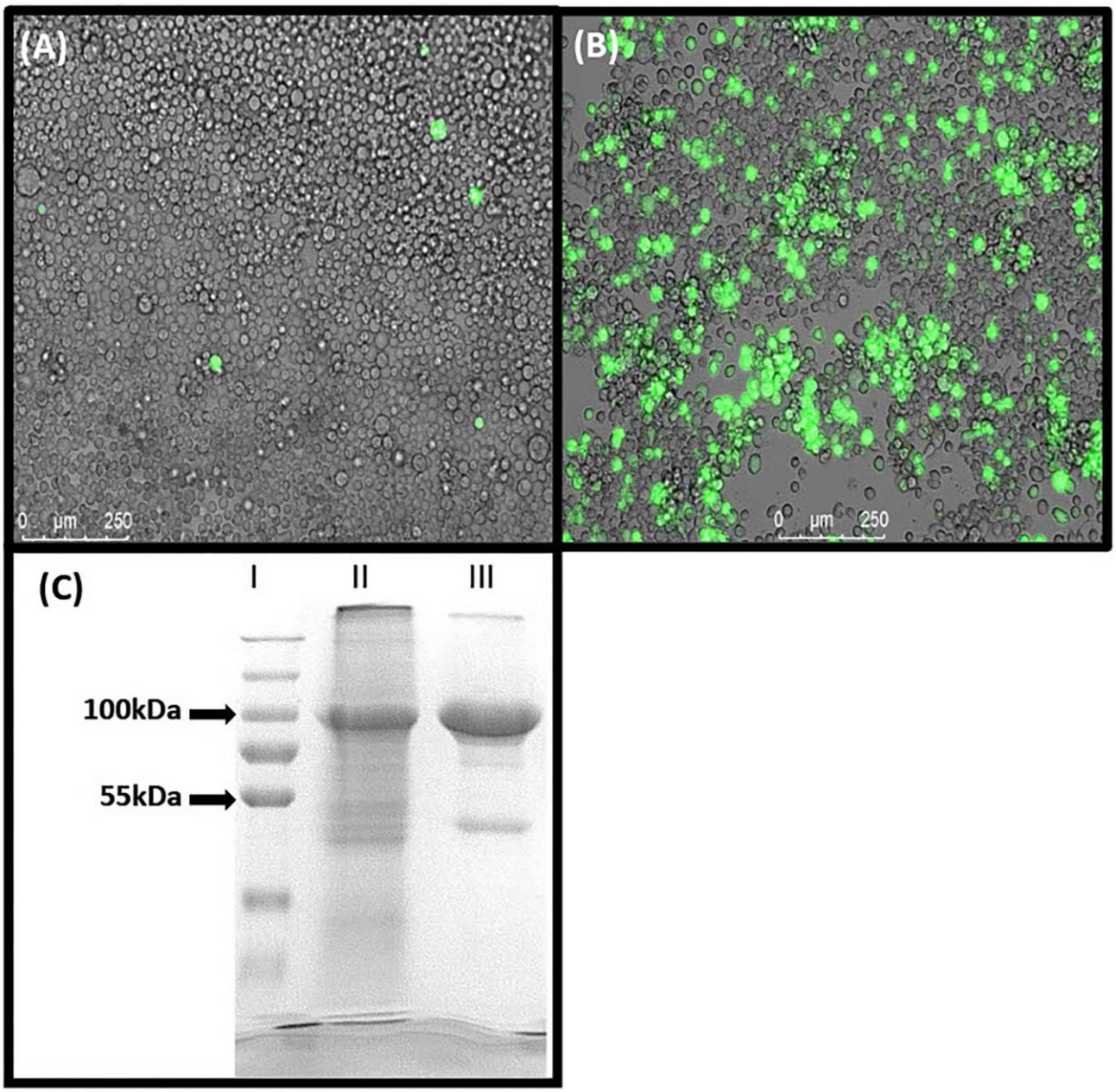

FIGURE 2 | Protein expression. (A) SF21 cells expressing eGFP after transfection with Bacmid DNA. (B) Hi5 cells expressing eGFP 1 day before harvesting. (C) SDS-PAGE of (I) Marker, (II) Lysate of Hi5 Insect cells overexpressing Amel_FTHFDH and (III) purified Amel_FTHFDH. Proposed specifically expressed recombinant protein appears at 100 kDa.

novo and need to ingest folate through their diet. Insufficient folate uptake can lead to deregulation of methylation processes (54), increased fragility of chromosomes due to decreased DNA repair capabilities (55, 56), and altered protein expression(57). If we assume that folate supplementation increases the detoxification capacity of 10-formyl tetrahydrofolate dehydrogenase, folic acid supplementation in the diet of honeybees could be used to increase the desired detoxification of formic acid.

In summary, we could show for the first time that recombinantly expressed enzyme 10-formyl tetrahydrofolate dehydrogenase of *A. mellifera* exhibits comparable activity to similar enzymes described in mammals with a Vmax value of 0.4253 nM min^{-1} at optimal pH. The confirmed activity of this specific enzyme implies a critical role in the detoxification of formic acid in the honeybee. In the future, better knowledge of this detoxicating enzyme may support honeybees' tolerance to the widely used formic acid for the treatment of varroa mites.

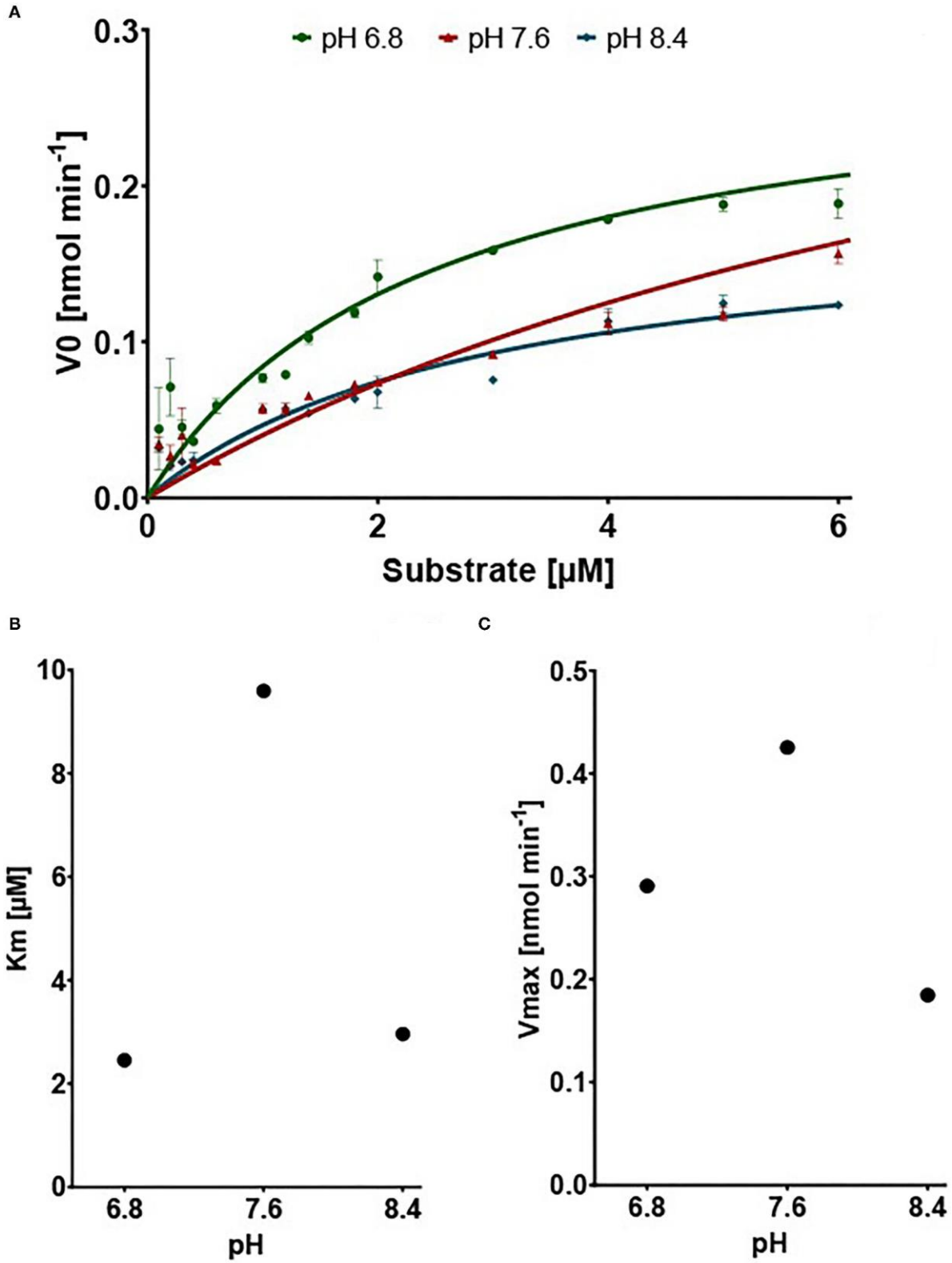

FIGURE 3 | Results of the dehydrogenase assays. (A) V_0 in nM min^{-1} in connection with increasing Substrate concentration. Points show mean of V0 replicates (n = 3) ±SEM (B) Vmax in nmol min^{-1} dependent on pH (C) Km in µM dependent on pH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MM designed the study, performed experiments, analyzed data, and wrote first draft of the manuscript. SS provided advice in study design and revised and edited the manuscript. RE provided advice in study design, revised and edited the manuscript, supervised planning, and execution. All authors read and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. Connector

The first study showed that the enzyme 10-FTHFDH is an important part of the detoxification mechanism of formic acid in *A. mellifera*. This enzyme catalyses the detoxification of formic acid similar to its mammalian counterparts. It utilizes the substrate 10-formyl tetrahydrofolate to transform it into tetrahydrofolate and CO_2, with similar kinetic constants of V_{max} and Km of 0.043 µmol min−1 mg−1 of enzyme and 9.6 µM, respectively. Due to poor conservation of the enzyme in comparison to well-described mammalian equivalents, the mode of action of the enzyme remains to be fully determined. Therefore, the second part of the project aimed to confirm the active site of the enzyme and elucidate the mode of action of the enzyme. To do this, a two-step approach was chosen. Firstly, the structure of the enzyme was resolved using *in silico* approaches involving different bioinformatic approaches. Thereafter, the retrieved structural model of the enzyme was used to perform *in silico* docking to predict the residues involved in the chemical reaction. Glutamate E673 and cysteine C707 are hydrogen bonded. Thereafter the glutamate sidechain is rotated by the binding of $NADP^+$ which leads to a greater distance between the glutamate and the cysteine. Now the negatively charged sulphur of the cysteine becomes transient covalently bound to the $NADP^+$s C4 atom of the nicotinamide ring. The cysteine performs nucleophilic catalytic functions, whereas the glutamate activates a water molecule (Tsybovsky & Krupenko, 2011). With the discovered active site and involved residues, *in vitro* mutagenesis was performed to experimentally verify the residues utilized within the enzyme. These findings provide in depth knowledge of the enzyme and are important for understanding the detoxification mechanism and therefore finding optimization possibilities for the formic acid treatment against *V. destructor*, which heavily impacts honey bee survival.

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The Active Site of the Enzyme 10-Formyl-THFDH in the Honey Bee *Apis mellifera*—A Key Player in Formic Acid Detoxification

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Abstract: Honey bees are important managed pollinators that fulfill important ecological and economic functions. In recent decades, the obligate ectoparasite *Varroa destructor* severely affected the survival of honey bees, as it weakened them by different means. A common treatment against *V. destructor* is formic acid fumigation, which has been used for decades by beekeepers across the world. This treatment is known to be effective, but many beekeepers report adverse effects of formic acid on bees, which include damage to the brood, worker bee mortality, and queen loss. Little is known about the molecular mechanisms of formic acid detoxification in honey bees. Recently, we reported upregulation of the bee enzyme, 10-formyl-THFDH, under formic acid fumigation. Here, the active site of this enzyme is characterized by an interdisciplinary approach combining homology modeling and protein mutagenesis. In addition, the limitations of the 3D protein structure prediction program AlphaFold2 are shown in regard to docking studies. This study provides a more thorough understanding of the molecular detoxification mechanisms of formic acid in *Apis mellifera*.

Keywords: *Apis mellifera*; 10-formyl-THFDH; mutagenesis; formic acid; detoxification; honey bee

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1. Introduction

Honey bees are known to play a key role in our food production and economy [1,2]. Since the vast majority of plants rely on pollination by bees [3] and crops are the basis for food and feed production, the rapid decline in honey bee colonies in recent decades [4,5] is worrying and of great concern not only to beekeepers but to the public in general. Various factors, owing to malpractices by beekeepers, increased use of pesticides by farmers, the emergence and prevalence of pathogens, and the mite *Varroa destructor* [6], play a key role in colony collapses [7,8]. *V. destructor* as an obligate ectoparasite, which feeds on the fat body and hemolymph of larvae and adult bees [9], directly affects their health. In addition, *V. destructor* is known as a vector of various pathogens, including viruses, such as deformed wing virus and chronic bee paralysis virus [10,11], as well as bacterial pathogens such as *Paenibacillus larvae* [12].

Organic acids such as formic acid are among the most important substances for treatment against *V. destructor* infestation. Due to its low risk of leaving residues in bee products, when applied correctly [13,14], it is licensed for the use in most parts of the world, including the EU, the USA, Canada, and most of Latin America [15–20]. Another positive aspect is that, so far, there is no known development of resistance to this treatment. Owing to the many different factors influencing the efficiency of the treatment, such as humidity, temperature, or type and placement of applicator, the treatment with formic acid often includes adverse effects [8,16]. Even though this treatment has been used by beekeepers for decades, its mode of action and the molecular basis of detoxification in honey bees are mainly unknown. Two of our recent studies showed transcriptional upregulation of the mRNA of the enzyme 10-formyl tetrahydrofolate dehydrogenase (10-formyl-THFDH) in honey bees treated with formic acid [21] and the detoxification capability of the recombinantly expressed enzyme [22]. The most recent study also revealed that the enzyme itself is poorly conserved between *Apis mellifera* and the well-described mammalian

orthologs. The overall conservation is found to lie between 55% and 70%, whereas the conservation within mammals is found to be higher than 90%. This raises the question of whether the enzyme utilizes the same active sites for the conversion of its substrate.

The ability of 10-formyl-THFDH to completely oxidize 1C units to CO_2 in an $NADP^+$-dependent reaction could remove formic acid from the organism [23]. As reported for mammals, the folate-dependent one-carbon pool (C1) is the most important detoxification pathway of formic acid, catalyzing the conversion of tetrahydrofolate (THF) to 10- formyl-tetrahydrofolate (10-formyl-THF) by a 10-formyltetrahydrofolate synthase [24]. Subsequently, the aforementioned 10-formyltetrahydrofolate dehydrogenase catalyzes the $NADP^+$-dependent reaction of 10-formyl-THF to CO_2 and THF [25,26].

In 2021, the field of protein biochemistry was revolutionized by the AI program AlphaFold2, which allows for atomic close predictions of folded proteins solely based on their primary amino-acid sequence, even if no similar structure is known [27]. AlphaFold2 can be used for different applications, such as the design of expression constructs, de novo protein design, and 3D structure solution. As a result, time-consuming methods for molecular prediction, such as nuclear magnetic resonance (NMR), can be replaced by such machine learning-based approaches. Nevertheless, for complex interactions in and between large macromolecules, such as the interaction between RNA and proteins and in-between proteins, experimental techniques, including but not limited to NMR and X-Ray crystallography, can be applied [28,29]. These laboratory techniques are also the basis for a commonly used approach for structural prediction based on homology modeling. First structures of the target protein are often derived by either SWISS-MODEL [30] or I-TASSER [31]. Thereafter, the 3D model of a target is predicted using the 3D structures of known related proteins, which have been resolved experimentally. These compositions are used to determine the folding and the orientation of sidechains of amino acids. The structural prediction approach is based on evolutionary relationships, in which enzymes that are closely related are expected to have a similar folding [32].

Because our enzyme of interest belongs to the group of enzymes utilizing cofactors ($NADP^+$), we decided to characterize the interacting sites using an established approach [33]. By combining homology modeling and docking, with mutagenesis of the identified active site, the importance of the main amino acids essential for the enzymatic reaction can be confirmed. Furthermore, we aimed to compare the conservative method with the new AlphaFold2 algorithm in terms of the quality of structural prediction. Lastly, this work pursues the goal of identifying the reactive residues of this detoxifying en- zyme in the enzyme of *A. mellifera* to enable future targeted applications for the control of *V. destructor*.

2. Results

2.1. Homology Modeling, Docking, and Sequence Alignment of Wildtype Enzyme

The consensus sequence (Accession: XP_006563851.2) of 10-formyl-THFDH was used as a basis for the homology modeling with the 10-formyl-THFDH of *Rattus norvegicus* (Accession: NP_071992.2) for structure and side chain orientation, as the structure of the rat ortholog has been solved by crystallography [34].

To examine the conservation of this enzyme within the family of *Apidae* and between mammals and *Apidae*, a multiple-sequence alignment was performed using ClustalΩ [35]. The alignment of the resulting 17 sequences revealed that the active site is very well conserved with only minor exchanges (Figure S2). The key residues and their surrounding regions are conserved with a 100% fit.

The homology modeling and docking revealed a catalytic subdomain and a $NADP^+$-binding subdomain with its $NADP^+$-binding site and a substrate entrance tunnel (Figure 1A). The docking also revealed a binding pocket of the substrate 10-formyl-THF with a negatively charged cavity, which is capable of binding the positively charged region of the substrate (Figure 1B,C). Two interacting amino-acid residues were predicted in the enzyme: E673 and C707. Subsequently, both residues were mutagenized to validate their function.

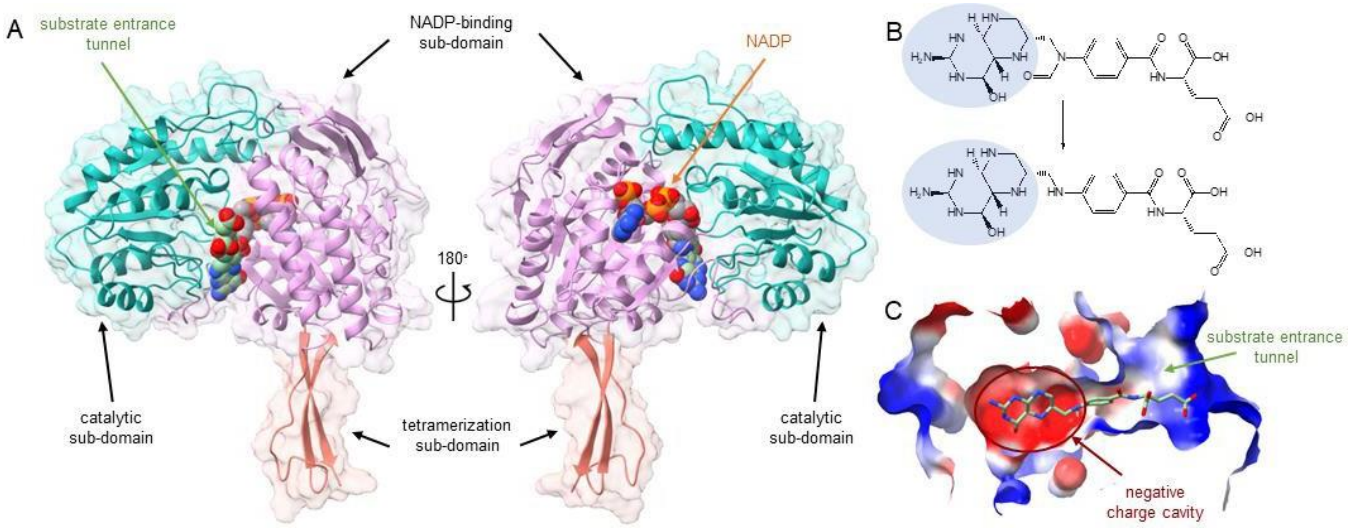

Figure 1. (**A**) The C-terminal structure of 10-formyl-THFDH. The C-terminal 10-formyl-THFDH domain includes the catalytic subdomain (turquoise), the $NADP^+$-binding subdomain (light pink), and the tetramerization subdomain (salmon). On the left is the substrate entrance tunnel. The substrate (10-formyl-THF) is shown in light green. The $NADP^+$-binding site is opposite to the substrate-binding site. On the right is the structure rotated 180°. $NADP^+$ is shown in gray, Oxygen atoms are shown in red, and nitrogen atoms are shown in blue. The substrate carbon atoms are shown in light green. $NADP^+$ carbon atoms are shown in gray. (**B**) The substrate reaction. The highlighted areas of substrate and product show the positively charged region (for pKa-calculation see Figure S4). After the enzymatic reaction, the substrate is oxidated, and one molecule of CO_2 (not shown) is produced. (**C**) Clip of the substrate entrance tunnel with its negatively charged cavity to bind the substrate.

The superimposed structures of the apo-protein and the $NADP^+$-binding form re- vealed a closed loop in the apo-form, which sterically clashes with the substrate (Figure 2A). In the $NADP^+$-bound form, the loop opens and reveals the substrate entrance tunnel and the negatively charged cavity. Binding in this pocket allows the substrate to be covalently bound to the C707 which initiates multiple steps to fully oxidize the formyl group. After the formation of the thiohemiacetal, a hydrogen is donated to the $NADP^+$ cofactor which results in the formation of a thioester. The following step includes the addition of another hydroxy group, whereafter the E673 donates its electron to accept the hydrogen atom of before mentioned hydroxy group. In the following deacetylation step, a tetrahedral intermediate is formed, which allows the cysteine to break the bond, with the product resulting in the formation of a carboxyl group which is oxidized to CO_2 (Figure 2B,C).

2.2. Homology Modeling and Docking of Mutants

The homology modeling of the mutants was performed as described above. The amino-acid sequences were modified with punctual mutations of the critical residues identified by the docking of the wildtype enzyme with $NADP^+$ and the substrate. The following residues were exchanged: C707 to A707 (C707A), E673 to A673 (E673A), and E673 to D673 (E673D). The homology modeling and docking of the mutants with the substrate revealed that no covalent bond could be formed between the alanine mutation of the residue C707 and the substrate formyl group. The residue E673 is expected to activate a water molecule. The E673D exchange, providing the same reactive carboxyl group, is

expected to show similar binding capability compared to the wildtype, with the length shortened by one CH_2 molecule. The alanine exchange is expected to have lower binding capability as the exchange denies the ability to activate the water molecule. Interestingly, a steric clash is seen in all mutants between the substrate and the phenylalanine at position 870 of the enzyme (Figure S1A–D).

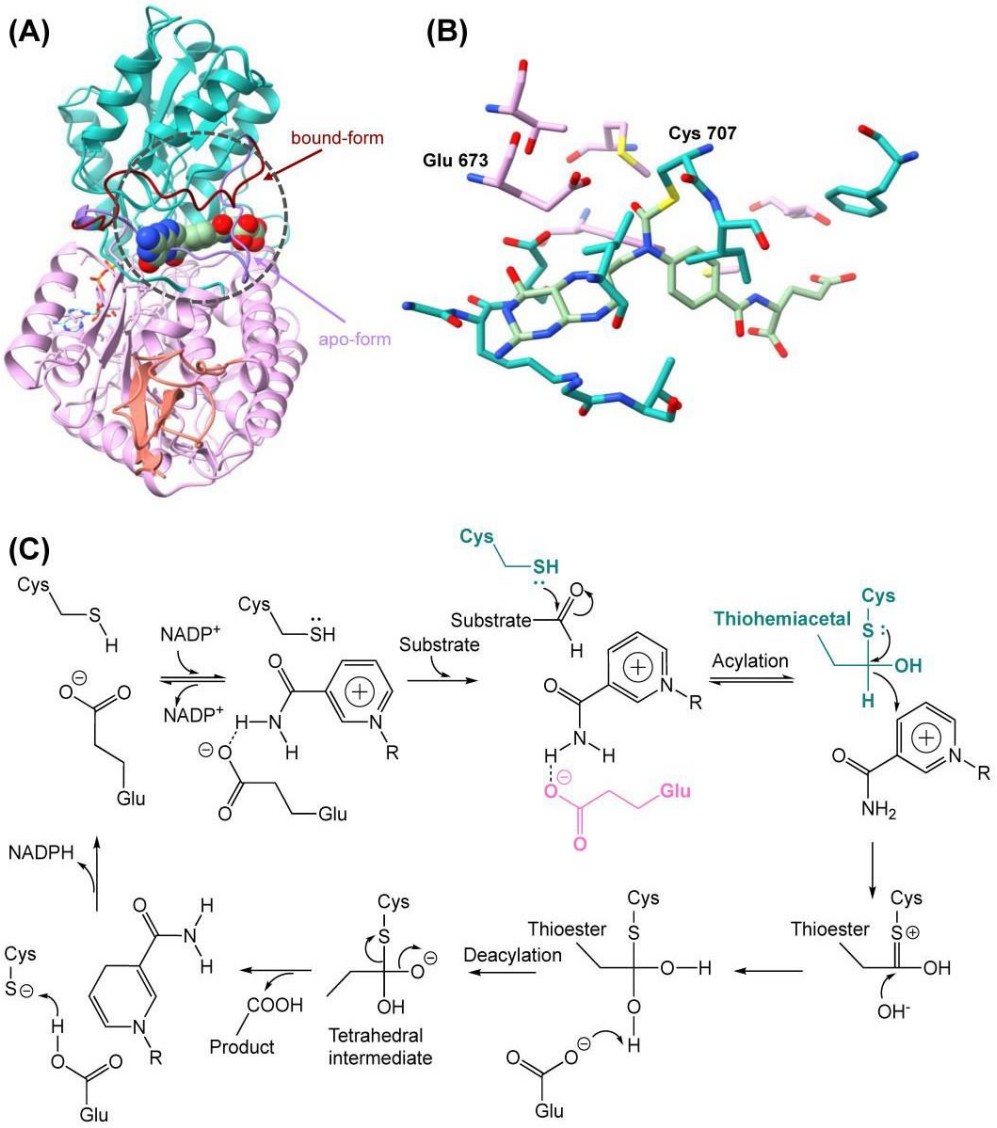

Figure 2. (**A**) The superimposed structure of apo-form and binding-form of 10-formyl-THFDH. The light purple loop is the apo-form. The red loop is the bound form. The substrate molecule is shown in ball representation. Substrate carbon atoms are shown in light green, oxygen atoms are shown in red, and nitrogen atoms are shown in blue. The apo-form loop shown in purple has a steric clash with the substrate molecule. Once the enzyme is activated by $NADP^+$, the loop is fully opened and forms the substrate entrance tunnel, and the substrate enters the binding pocket. (**B**) Zoomed-in view of the binding pocket of the substrate, which is covalently bound to the C707. (**C**) The 10-formyl-THFDH oxidation reaction mechanism. The lone pair of electrons of the sulfur in the thiol group of the cysteine nucleophilically attacks the oxygen from formyl group of the substrates.

2.3. Comparison of AlphaFold2 Model and Homology Models

The consensus sequence (Accession: XP_006563851.2) of 10-formyl-THFDH was subjected to the AlphaFold2 algorithm for structural prediction. The superimposed structures of the AlphaFold2 prediction and the apo-protein (Figure 3A) are both very similar. Overall,

both models showed a root-mean-square deviation of atomic position (RMSD) of 0.843 Å, which indicates close similarity.

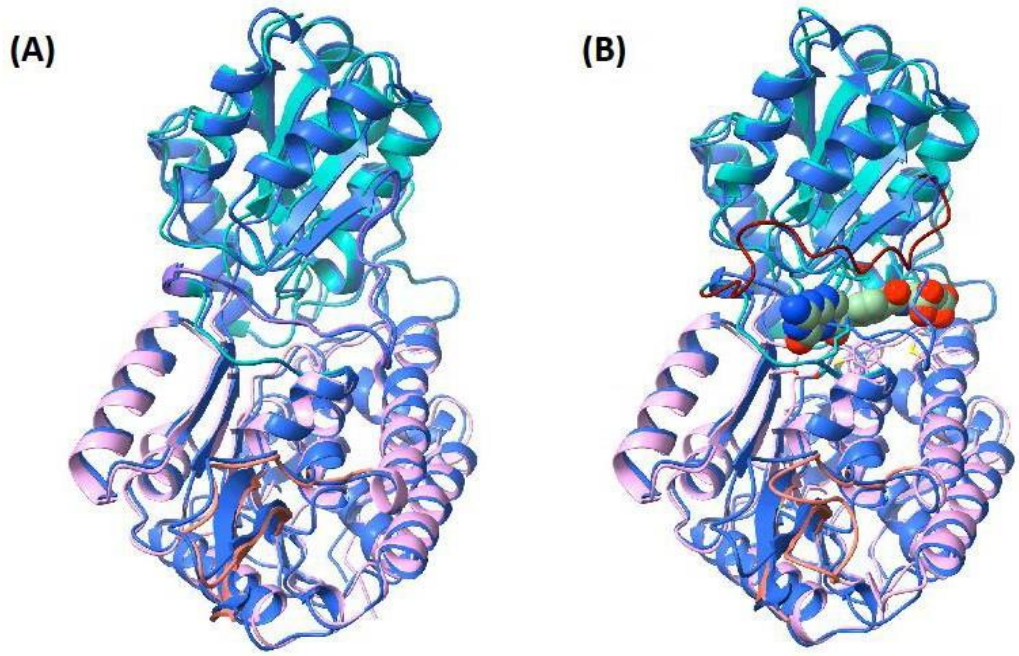

Figure 3. The superimposed models of the homology model and the AlphaFold2 model. (**A**) The AlphaFold2 model (blue) and the apo-form of the enzyme (teal: catalytic subdomain; pink: $NADP^+$-binding subdomain; orange: tetramerization subdomain). The RMSD of these two structures is 0.843 Å. The AlphaFold2 structure shows a similar loop to the apo-form homology model. (**B**) The superimposed view of the AlphaFold2 structure and the ligand-bound form of the homology model (teal: catalytic subdomain; pink: $NADP^+$-binding subdomain; orange: tetramerization subdomain; dark red: open loop). The RMSD of these two structures is 0.932 Å. The AlphaFold2 structure shows that the loop clashes with the ligand.

The superimposed structure of the AlphaFold2 model and the bound-form homology model differed in the loop position (Figure 3B). The overall RMSD was 0.932 Å. In the AlphaFold2 structure and in the apo-protein form, the loop crosses the ligand, preventing binding of the substrate to the active site.

2.4. Enzyme Kinetics

The aforementioned amino-acid exchanges of identified critical residues (C707A: Cys → Ala; E673D: Glu → Asp; E673A: Glu → Ala) were experimentally validated by means of site-directed mutagenesis of 10-formyl-THFDH of *A. mellifera*. All recombinant derivatives were successfully expressed and purified. The dehydrogenase activity was measured by monitoring the increase in absorbance at 340 nm. The recombinantly ex- pressed wildtype protein showed an enzyme activity with a Vmax of 0.1846 $nM \cdot min^{-1}$ to 0.4253 $nM \cdot min^{-1}$ at different pH and Km values of 2.455 µM up to 9.596 µM (according to previously published data [22]). The mutations lost their activity almost entirely, with values ranging between 0.007 $nM \cdot min^{-1}$ and 0.12 $nM \cdot min^{-1}$ for their Vmax values and between 8.33×10^{-14} µM and 8.57×10^{-16} µM for their Km values; additionally, it has to be stated that all R^2 values, indicating the goodness of fit, for the mutants were below 0.17, which indicates a poor fit of the used nonlinear regression (Figure 4, Table 1).

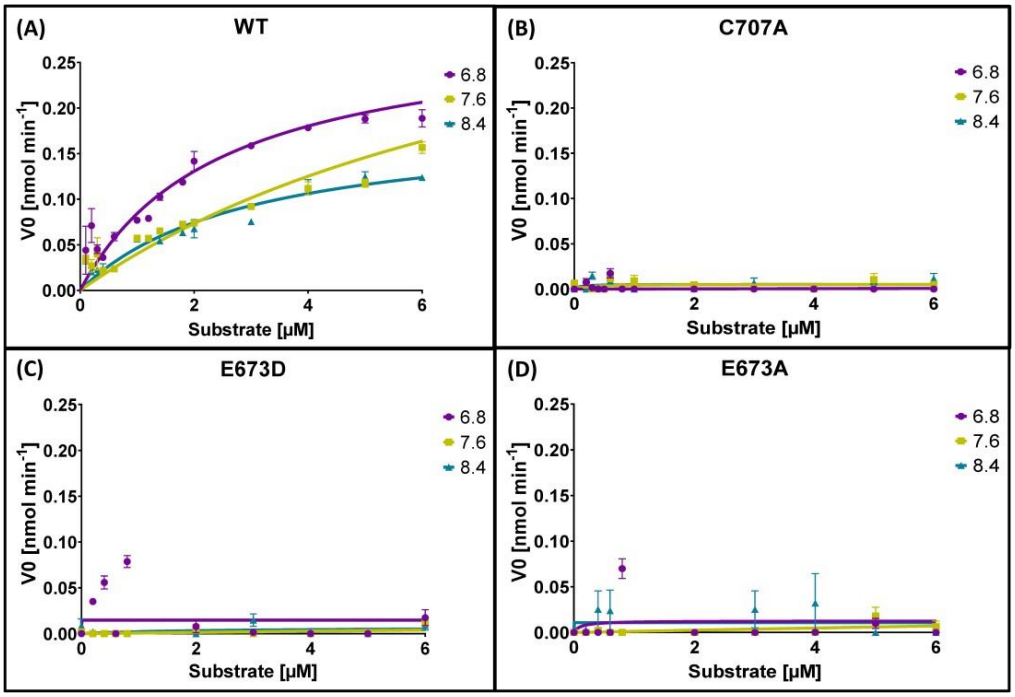

Figure 4. (**A**) Activity of the recombinantly expressed wildtype enzyme 10-formyl-THFDH. (B) Enzyme activity of the mutation with an alanine exchange at the cysteine 707 position (C707A). (C) Enzyme activity of the mutation with an aspartic acid exchange at the glutamic acid 673 posi- tion (E673D). (**D**) Enzyme activity of the mutation with an alanine exchange at the glutamic acid 673 position (E673A).

Table 1. Vmax and Km values with statistics. The calculated Vmax and Km values for each mutant 10-formyl-THFDH (including wildtype (WT)), as well as their respective 95% confidence interval and the goodness of fit as R^2.

pH	Enzyme	V_{max}	K_m	95% CI V_{max}	95% CI K_m	R^2
6.8	WT	0.2907	2.455	0.2515 to 3.542	1.72 to 3.542	0.8290
	C707A	NA	8.57×10^{16}	NA	NA	−0.1333
	E673A	0.01265	0.1109	0.0026 to 1.762×10^{7}	NA	0.0207
	E673D	0.01484	8.33×10^{-14}	0.0078 to 0.02894	NA to 0.2658	0.0262
7.6	WT	0.4253	9.596	0.2908 to 83.76	5.133 to 23.98	0.8858
	C707A	0.00538	0.228	0.0023 to 0.01327	NA to 4.951	−0.0632
	E673A	0.1299	102.8	0.0037 to NA	0.2755 to NA	0.1713
	E673D	0.05799	81.54	0.0021 to NA	NA	0.1493
8.4	WT	0.1846	2.961	0.1520 to 0.2346	1.934 to 4.670	0.9029
	C707A	0.005269	0.09273	0.0025 to 2.612×10^{7}	NA	0.0408
	E673D	0.006758	2.156	0.0018 to 1.634×10^{9}	NA	0.0050

3. Discussion

Natural colonization of floral nectar by yeast and bacteria [36–39] often results in the production of methanol and ethanol by fermentation [40,41]. Hence, bees need to be able to detoxify formic acid, as it is the main driver of methanol toxicity [42]. The strong conservation of the enzyme within the family of *Apidae* (Figure S2) underlines the importance of the protein characterized here, which exhibits 100% conservation of the active residues E673 and C707 and the surrounding region, even in the relatively distinct genera *Eufriesea* and *Apis* [43] and between Mammalia and *Apidae*.

The folate-dependent one-carbon pool (C1) is known to be the major detoxification pathway of formic acid in mammals and presumably also in *A. mellifera*, catalysing the conversion of THF to 10-formyl-THF by a 10-formyl-THF synthase [24]. Subsequently, the aforementioned 10-formyl-THFDH catalyzes the $NADP^+$-dependent reaction of 10-formyl- THF to CO_2 and THF [25,26]. This highlights the importance of folic acid as one of the main factors influencing the efficiency of this detoxification mechanism, as it is the first substrate in the reaction chain. A dietary supplementation might be beneficial for the honey bee to optimize survival during formic acid treatments. On the basis of our molecular data, further research in this area is now being encouraged, e.g., to better understand the detoxification of formic acid used against *V. destructor* in honey bees in the field. The next aim is to use this knowledge for developing better and more bee-friendly formic acid treatment strategies.

AlphaFold2 is an incredible advance in the protein modeling world. This study shows how close AlphaFold2 structural predictions come to the more work-intense conservative methods, such as homology modeling. The difference between the apo-form enzyme and the AlphaFold2 model is almost negligible with an RMSD of 0.843 Å. With a difference of 0.932 Å between the bound-form homology model and the AlphaFold2 prediction, the model also appears to be well suited for docking models. This is a misconception, because the main difference in the RMSD is in the most important part, the loop that blocks the substrate entrance tunnel in the apo-form (Figure 2A). This cannot be correctly predicted, as AlphaFold2 is unable to include co-factor binding in its prediction (Figure 3B). Additionally, the "dark proteome", which is estimated to comprise 44–54% of all proteins in eukaryotic cells, consists of proteins that are unstably folded and, therefore, have no well-defined three- dimensional structure [44,45]. Such proteins are thought to play a role in defense or signal transduction and change their structure when interacting with different macromolecules such as RNA and other proteins. Another example where AlphaFold2 is currently reaching its limits are enzymes with co-factors. In these enzymes, a conformational change is initiated, which in turn enables the enzyme to bind its specific substrate to trigger further reactions [28]. AlphaFold2 seems to be unable to predict loop changes caused by the binding of before mentioned cofactors. This can lead to problems in the prediction of binding sites, as shown in our example.

Despite the low conservation of the enzyme 10-formyl-THFDH between mammals and *A. mellifera,* this study shows that the active site of the dehydrogenase subunit remains almost unchanged. On one hand, Tsybovsky and Krupenko [46] have already shown that the residues C707 and E673 are important for the degradation of 10-formyl tetrahydrofolate in *Rattus norvegicus*. However, on the other hand, the conservation of this enzyme between the two species *A. mellifera* and *R. norvegicus* is so low [22] that confirmation of the active site is necessary to make further functional statements. The expected molecular mode of action is as follows: first, glutamate E673 and cysteine C707 are hydrogen-bonded. The binding of $NADP^+$ results in the rotation of the glutamate sidechain away from the cysteine; thereafter, the negatively charged sulfur of the cysteine forms a transient covalent bond with the C4 atom of the nicotinamide ring of the $NADP^+$. In the two phases of the dehydrogenase catalysis, acetylation and deacetylation, the cysteine fulfills a nucleophilic catalytic function, whereas the glutamate is expected to activate a water molecule in the deacetylation step [46]. With the proposed mechanism, the two mentioned residues are of great importance. The mutations showed no activity in comparison to the wildtype enzyme. Additionally, the homology modeling and docking revealed one main problem with the binding site of the substrate, when one of the key residues was changed. The steric clash between the substrate and F870 of the enzyme seemed to result in almost no activity in all mutants investigated (Figure S1B–D). A double mutation of the wildtype, involving a mutation of the phenylalanine and the E673 residue of the enzyme, could shed light on whether this steric clash is the reason why the glutamic acid for aspartic acid exchange(E673D) loses its activity completely, although only a decrease in activity is to be expected, since aspartic acid and glutamic acid are similar in their biochemical properties.

In conclusion, our approach allowed verification of the active site of honeybee 10-formyl-THFDH, although only evolutionary very distant enzymes have been experimentally confirmed so far. In addition, the importance and detoxifying potential of the honey bee 10-formyl-THFDH was demonstrated at the molecular level, which would also be expected for other species in the family of *Apidae*. At the same time, this study points to certain limitations of the currently used AlphaFold2 algorithm.

4. Materials and Methods

4.1. Homology Modeling

The homology models were built by Prime [47]. The ligand-bound form loop of the homology model was built by Prime (Schrödinger Release 2019-4).

4.2. Covalent Docking

The homology model was used to generate the receptor grid. The substrate was prepared by LigPrep. The force field was OPLS3 [48]. The covalent docking method from Glide was used to dock the substrate into the receptor [49].

4.3. AlphaFold2 Structural Prediction

The full-length 10-formyl-THFDH predicted structure was obtained by AlphaFold2 Python code on Google Colab (Figure S3) (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb, accessed on 14 April 2022) [27].

4.4. Structural Analysis

The electrostatic surface of the binding pocket was analyzed by ChimeraX. The RMSD values between two different structures were calculated by PyMOL (Version 2.4.2 Schrödinger, LLC.) [50,51].

4.5. Visualization

All modeling figures were processed and presented by ChimeraX [50,51].

4.6. Multiple Sequence Alignment

ClustalΩ [35] was used for amino-acid sequence alignment. Thirteen consensus sequences of different species of the family *Apidae* from across the globe and selected mammalian species were used (Table S1).

4.7. A. Mellifera Sampling

One day old worker bees were collected from the apiary of the Institute of Veterinary Biochemistry, Freie Universität Berlin, Berlin (52.42898° N, 13.23762° E) using one queen- right colony with *A. mellifera* (carnica) in the summer season 2020. Colonies were healthy, had enough food supply, and showed no symptoms of diseases or increased parasitism. Individuals were shock-frozen in liquid nitrogen and stored at −80 °C until further use.

4.8. RNA Extraction

RNA extraction was performed using the Quick-RNA™ Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany). Briefly, individuals were lysed in a lysing Matrix S (MP Biomedicals, Heidelberg, Germany) containing 1 mL of lysis buffer using a BeadBlaster (Benchmark Scientific, Edison, NJ, USA). Tubes were then centrifuged at 12,000× *g* at 4 °C for 10 min. The supernatant was transferred into a clean microcentrifuge tube containing 1× volume of 100% ethanol. The solution was then used according to manufacturer's protocol. RNA was eluted in a total volume of 50 µL of ddH_2O. The quantity and quality of total RNA were analyzed using an Agilent RNA 6000 nano chip on a 2100 Bioana-

lyzer (Agilent Technologies, Santa Clara, CA, USA). Isolated RNA was stored at −80 ˚C until use.

4.9. First-Strand cDNA Synthesis

Protoscript® II Transcriptase (New England Biolabs, Inc., Ipswich, MA, USA) was used according to the manufacturer's protocol. Briefly, 1 µg of DNA-free RNA was incubated with 1 µL of d(T)23VN-Primer (50 µM) and 1 µL of Random Primer Mix (50 µM) at 65 ˚C for 5 min in a total volume of 8 µL. Thereafter, 12 µL of Protoscript Mastermix was added, and the sample was incubated at 42 ˚C for 60 min and heat-inactivated at 80 ˚C for 5 min. The cDNA was then diluted by the addition of 80 µL of ddH_2O and stored at −20 ˚C in adequate aliquots. To create a broad library, 5 µL of each sample was added to one microcentrifuge tube before freezing.

4.10. Creation of pFBD-eGFP-Amel_10-Formyl-THFDH Expression Vector

The open reading frame of the wildtype *A. mellifera* cytosolic 10-formyl-THFDH (Ac- cession: XM_026442355.1) (Amel_10-formyl-THFDH) was amplified by polymerase chain reaction (PCR) using the primers Amel_FTHFDH_ORF_F/R along with the full-length cDNA (Table 2). The PCR product was then subcloned into pJet1.2 vector (Thermo Scien- tific, Karlsruhe, Germany) for sequencing and creation of a template for further use. The ORF-containing vector was used to create overhangs containing restriction sites (BamHI, NotI) and a 6×-HisTag at the N-terminus for later purification of the protein. The pFastBac- Dual (pFBD) vector of the Bac-to-Bac System (Thermo Scientific, Karlsruhe, Germany) with an enhanced green fluorescent protein (eGFP) cloned at the p10-promoter site was used as expression vector. The insert was created by PCR using the Amel_FTHFDH_BHI_HT_F and Amel_FTHFDH_NotI_R primers (Table 2). pFBD-eGFP was digested with appropriate restriction enzymes, and the vector was dephosphorylated using an Antarctic Phosphatase (New England Biolabs, Inc., Ipswich, USA) to prevent religation. The PCR product was ligated with the vector using a T4-ligase (New England Biolabs, Inc., Ipswich, USA) according to standard protocols.

Table 2. Primer used for vector creation and mutagenesis.

Name	5′–3′ Sequence
Amel_FTHFDH_ORF_F	ATGGCGCAACTCAAAGTGGC
Amel_ FTHFDH _ORF_R	CTAATATTCTACAGTGATAGTTTTTG
Amel_ FTHFDH _BHI_HT_F	TCATACGGATCCATGCACCACCACCACCACCACGCGCAACTC AAAGTGGC
Amel_ FTHFDH _NotI_R	TCATACGCGGCCGCCTAATATTCTACAGTGATAGTTTTTG
Amel_ FTHFDH _E673A_F	ATCCCTAGCATTAGGTGGAA
Amel_ FTHFDH _E673D_F	ATCCCTAGACTTAGGTGGAA
Amel_ FTHFDH _E673AD_R	ACTTTCTTCAAATTACTATT
Amel_ FTHFDH _C707A_Ass_F	CAAAGGAGAAAACGCAATAG
Amel_ FTHFDH _C707A_Ass_R	CTATTGCGTTTTCTCCTTTGTTGAAGAACAC

4.11. Mutagenesis of the Expression Vector

For the mutagenesis of the expression vector and creating the three mutants (C707A, E673D, and E673A), two different approaches were used. Firstly, the KLD enzyme mix from New England Biolabs was used to create the expression vectors E673D and E673A according to the manufacturer's protocol using the primers pFBD_Amel_FTHFDH_E673A_F/R and pFBD_Amel_FTHFDH_E673D_F/R (Table 2). For the creation of the expression vector for the mutant C707A, an assembly PCR approach was used [52], due to the KLD mix not work- ing for this mutant. Briefly, Amel_FTHFDH_C707A_Ass-F/R, Amel_FTHFDH_BHI_HT_F, and Amel_FTHFDH_NotI_R primer (Table 2) were used to create partly

overlapping ampli- cons, which were thereafter assembled to create the full-length ORF with the appropriate mutation. Site-directed mutagenesis was performed using 50 ng of purified template and a Q5-Polymerae (NEB). After the initial step at 98 ˚C for 5 min, the first 10 cycles were run for 30 s at 98 ˚C and 20 s at 56 ˚C + dT of 0.5 ˚C per cycle, followed by 1.5 min at 72 ˚C. The next 30 cycles were conducted according to the following scheme: 30 s at 98 ˚C, 20 s at 61 ˚C, and 1.5 min at 72 ˚C, followed by a terminal step at 72 ˚C for 3 min. Amplicon assembly

was performed using a two-step protocol. First, equimolar amounts of both gel purified amplicons were added to the reaction containing 1 U of Q5-Polymerase, dNTPs, and 10× buffer in 25 µL of total volume. The assembly was started at 98 ˚C for 5 min, followed by 10 cycles with 30 s at 98 ˚C, 20 s at 61 ˚C, and 1.5 min at 72 ˚C. The reaction was cooled down to 4 ˚C, and primers Amel_FTHFDH_BHI_HT_F and Amel_FTHFDH_NotI_R were added. Thereafter, the amplification was performed using the following protocol: 98 ˚C for 5 min, followed by 30 cycles of 98 ˚C for 30 s, 61 ˚C for 20 s, and 72 ˚C for 2 min, followed by a terminal step at 72 ˚C for 5 min. The mutated insert was inserted to the expression vector as described under Section 2.4.

4.12. Creation of the Recombinant Bacmid

To create the recombinant Bacmid, Gibco™ Max Efficiency™ DH10Bac competent cells (Thermo Scientific, Karlsruhe, Germany) were transformed using 1 µg of pFBD construct. The cells were thawed on ice, and 1 µg of construct was added. The mixture was incubated for 30 min on ice, heat-shocked for 45 s at 42 ˚C, and transferred back to ice for 2 min. Then, 900 µL of SOC medium was added. The culture was incubated for 4 h at 37 ˚C in a shaking incubator at 225 rpm. The cells were plated on LB medium containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10µg/mL tetracycline, 500 µg/mL X-Gal. and 1 µM IPTG. The plates were incubated for 48 h at 37 ˚C. White colonies were restreaked, and Bacmid was isolated according to the manufacturer's protocol.

4.13. Creation of Baculovirus

To create recombinant baculovirus, Sf21 insect cells (Thermo Scientific, Karlsruhe, Germany) were transfected with 1 µg of Bacmid DNA. Then, 6 µL of Gibco™ Cellfectin™ II reagent (Thermo Scientific, Karlsruhe, Germany) was used as suggested by the manu- facturer. Successful transfection was monitored by expression of eGFP under an inverse fluorescent microscope DMI 6000B (Leica), and photos were taken using a DFC 365FX (Leica) camera. Virus stock was extracted by detaching cells from flask and centrifuging at 3000× *g* for 5 min. Virus containing supernatant was transferred into a sterile 15 mL centrifuge tube and stored safe from light at 4 ˚C until further use.

4.14. Expression and Purification of Recombinant Proteins

To produce recombinant protein, Hi5 cells (Thermo Scientific, Karlsruhe, Germany) at 80–90% confluency were used. A total of 3×10^6 cells were seeded into a T175 flask (Sarstedt) containing 50 mL of Gibco™ ExpressFive™ SFM (Thermo Scientific, Karl- sruhe, Germany). Then, 30 µL/mL Virus-Stock was added, and cells were incubated at 27 ˚C for 4 days or until most cells showed eGFP expression. Cells were pelleted at 5000× *g* for 20 min at 4 ˚C. Pellets were resuspended in 20 mL of PBS containing EDTA-free proteinase inhibitor cocktail (SIGMA-ALDRICH, Darmstadt, Germany). The suspension was sonified on ice using a sonifier 250 (Branson) for 4 min with an amplitude of 2 at 20% energy. The

suspension was cleared by centrifugation at 5000× *g* for 20 min at 4 °C. Protein-containing supernatant and PBS containing imidazole at different concentrations (10 mM (equilibration buffer); 25 mM (wash buffer); 100 mM, 150 mM, 200 mM, and 500 mM (elution buffer)) were particle-free filtered (0.45 µM pore size, PES). Next, 2 mL bed-volume (BV) of HisPur™ Ni-NTA Resin (Thermo Scientific, Karlsruhe, Germany) was equilibrated with 5 BV of equilibration buffer. The protein was equilibrated with 20 mL of equilibration buffer and added to the column. The column was washed with 20 BV of wash buffer; thereafter, four elution fractions were obtained using four different concentrations of imidazole (100 mM, 150 mM, 200 mM, and 500 mM). The whole purification was per- formed at 4 °C. Thereafter, to remove impurities and imidazole from the enzyme, protein concentrators with a molecular weight cutoff of 50 kDa (Pierce) were used as suggested by the manufacturer.

4.15. Synthesis of 10-Formyl-THF

To synthesize the substrate 10-formyl-THF, an established protocol by Rabinowitz et al. [53] was used. Briefly, 100 mg of dl-5-formyltetrahydrofolic acid (SIGMA-ALDRICH, Darmstadt, Germany) was dissolved in 8 mL of 1 M β-mercapto-ethanol (Roth). The pH was adjusted to 1.5 with HCl. The mixture was stored at 4 °C for at least 12 h. The solution containing dl-5,10-methenyltetrahydrofolic acid as a precipitate (bright yellow tint), was adjusted to pH 8 with KOH, purged with argon, and incubated overnight at 4 °C in an evacuated vessel. The final solution containing 10-formyl-THF (clear color) was directly used for enzyme assays.

4.16. Enzyme Activity Assays

All assays were performed using a ClarioStar plus multimode plate reader (BMG labtech). All mutants were tested with the same batch of synthesized substrate. First, 100 mM β-mercapto-ethanol, 200 µM $NADP^+$, and 10 µg of purified enzyme were added to each well and incubated at 30 °C for 2 min. The substrate was injected using built- in injectors at different concentrations. NADPH production was monitored at 340 nm for a period of 30 min. All substances were diluted in Tris/HCl buffer (pH 6.8–8.4) to a total of 100 µL. The K_m and V_{max} were calculated using a molar extinction coefficient of 6220 $M^{-1} \cdot cm^{-1}$ for NADPH.

4.17. Analysis of Kinetic Data

Initial reaction rates were used to determine the respective enzyme activities. Ki- netic parameters were derived using GraphPad Prism version 9.0.2 (for Windows 10, GraphPad Software, San Diego, CA, USA, www.graphpad.com, accessed on 3 March 2022), which determined the kinetic parameters from the Michaelis–Menten equation using nonlinear regression.

Supplementary Materials: The following supporting information can be downloaded at: https:

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6. Discussion

These studies show that, the predicted 10-formyl tetrahydrofolate dehydrogenase exhibits very similar activity to known mammalian counterparts, as well as the verification of the active site of the enzyme. Therefore, it is very likely involved in the detoxification process of formic acid in the honey bee *Apis mellifera*. Both of the studies extensively discuss how the 10-FTHFDH of *Apis mellifera* compares to known equivalents in regards to activity and residue utilization in the active site of the enzyme.

6.1. Impact on formic acid treatment protocols for *Apis mellifera*

This newly obtained knowledge is very valuable as it gives in depth insight into the detoxification process of formic acid, but also draws similarities between *A. mellifera* and mammals and therefore might be used to optimize the formic acid treatment. Formic acid is the main driver behind methanol poisoning in mammals (Barceloux et al., 2002), as methanol is converted by the metabolism to formic acid which leads to acidosis and the inhibition of mitochondrial cytochrome oxidase (Liesivuori et al., 1991). Even though in this example formic acid is directly involved in a poisoning process, it is used as a treatment against *Varroa destructor* infestation, where these mites abundancy is greatly reduced and the potential of the spread of pathogens is heavily reduced. The parallels to the known mechanism of methanol poisoning and the involved formic acid opens the door for optimization in formic acid treatments of the honey bee hives. The management guideline of the American Academy of clinical Toxicology (Barceloux et al., 2002) states that methanol poisoning and therefore the treatment of formic acid poisoning is achieved by standard care, correction of metabolic acidosis, the administration of folinic acid, provision of an antidote and selective haemodialysis. As one can imagine some of those applications are simply impractical when it comes to managed honey bees. For example, a standard care protocol, which includes checking for symptoms, drug testing and infusions is simply not applicable for each individual honey bee. On the other hand, we could adapt the care for honey bees under formic acid fumigation by implementing a supplementation of (1) agents used for the correction metabolic acidosis, such as sodium bicarbonate; (2) folinic acid, being a bioactive form of THF and (3) an antidote, preferably fomepizole as competitive inhibitor of alcohol dehydrogenases. In general, a supplementation via feed (syrup or candy) is easily achieved and shown to be effective with different types of supplements, for example probiotics (Kaznowski et al., 2005), mushroom extracts (Stevanovic et al., 2018) and commercially available formulas like “HiveAlive” (Charistos et al., 2015). Overall the feeding of these supplements has been shown to increase hive strength and resilience against pathogens like *Nosema ceranae*. But still with all the aforementioned supplementations for treatment of formic acid poisoning further studies need to be conducted to inspect toxicity and benefit for *A. mellifera* as well as the influence on *V. destructor*, as no studies have yet been performed. The impacts on *V. destructor* survival especially need to be monitored, as we do not have a lot of insight on the molecular mechanisms of detoxification, except for the up regulation of the flavin-containing monooxygenase 5 (Genath et al., 2020) and the dysregulation in the proteome (Genath et al., 2021).

6.2. Potential further Application in *Apis mellifera*

This thesis also shows that the methodology used is suitable for providing in depth knowledge of detoxification processes and other pathways. By using a combination of *in silico* and *in vitro* methods it was shown that the enzyme 10-FTHFDH is involved in the detoxification process of formic acid in *A. mellifera*. Generally, speaking little is known about the molecular mode of actions of most toxins the honey bee is in contact with (Johnson, 2015). The molecular effects of many Xenobiotics are often described, here proteins of the superfamily Cytochrome P450 are involved in the detoxification, often very specific for certain Xenobiotics (Berenbaum & Johnson, 2015; Glavan & Bozic, 2013), for other pesticides such as monoterpenoids very little is known. For example, thymol directly impacts the function of octopaminergic system (Bonnafé et al., 2015), and the cholinergic system (Gashout et al., 2018) and causes dysregulation of genes important for dendrite morphogenesis, by the downregulation of the transcription factor *Pax 1*, the orthologue to *Poxm* in *Drosophila melanogaster*, and the resulting impact on memory formation and learning (Iyer et al., 2013; Paten et al., 2022), as well as the dysregulation V-type ATPases and mitochondrial and carboxylic transporters, impacting membrane permeability (Paten et al., 2022) For thymol as well as other organic acids (e.g. oxalic acid), as well as the proteins of *Bacillus thuringiensis,* commonly used as treatments against pests in plants, nothing in regards to the molecular mechanisms of detoxification in *A. mellifera* is known. The described methods would allow for the acquisition of in-depth knowledge of enzymes and their function with relatively small costs and facilities, while not being limited to insects.

7. Outlook

This thesis gave insight to parts of the detoxification mechanism of formic acid in *Apis mellifera,* where it is shown that 10-formyl tetra hydrofolate dehydrogenase is a key factor in the process. Further research should be focused on 3 main topics.

(1) Resolving the missing parts in the detoxification process in *Apis mellifera*, namely the functionality and structure of the upstream involved 10-formyl tetrahydrofolate synthase. This could be achieved with the same methodology showcased in this doctoral thesis.

(2) In field application of different supplements in bee hives under formic acid treatment, with the main focus on folinic acid, as it is the bioactive form of tetrahydrofolate, which is activated in the body. These experiments would be able to solidify the claims that a supplementation could bear benefits for the honey bees during formic acid treatment against *Varroa destructor*.

(3) The unravelling of the enzymes involved in detoxification of formic acid in *Varroa destructor*, as this might open new avenues to increase efficacy of formic acid treatment by combining possible inhibitors of the involved enzymes of *Varroa destructor* and the formic acid treatment.

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9. List of publications

Articles

Mating, M., Zou, Y., Sharbati, S., & Einspanier, R. (2023). The Active Site of the Enzyme 10-Formyl-THFDH in the Honey Bee Apis mellifera—A Key Player in Formic Acid Detoxification. International Journal of Molecular Sciences, 24(1), 354. https://doi.org/10.3390/ijms24010354

Mating, M., Sharbati, S., Einspanier, R. (2022). A Detoxification Enzyme for Apis mellifera Newly Characterized by Recombinant Expression: 10-Formyl Tetrahydrofolate Dehydrogenase. Front. Insect Sci. 2 (5). https://doi.org/10.3389/finsc.2022.829869

Presentations

Mating M. Cytosolic 10-THF of Apis mellifera – a key component in formic acid detoxification, Institut für Veterinär-Biochemie, Freie Universität Berlin, Berlin, 2021