ЖУРНАЛ СТРУКТУРНОЙ ХИМИИ

2007. Том 48, № 4 *Июль – август* *С. 666 – 673*

UDK 539.19:541.65

DFT STUDY OF THE OXALOACETIC ACID CONDENSATION — THE FIRST STEP OF THE CITRIC ACID CYCLE

© 2007 **V.B. Delchev[1]*, G.T. Delcheva[2]**

[1]*University of Plovdiv, Department of Physical Chemistry, Plovdiv, Bulgaria*
[2]*University of Food Technologies, Department of Biochemistry, Plovdiv, Bulgaria*

Received 29 November, 2006

The mechanism of acylation of oxaloacetic acid (OA) with acetyl—CoA was studied at the DFT level using basis functions 6-311G(*d*,*p*) and different numbers of diffuse functions. Four mechanisms were considered in this study. It was found that the most probable mechanism, in the approximation of isolated molecules, starts with the enol forms of oxaloacetic acid and acetylcysteamine (final fragment from acetyl—CoA). The mechanisms were commented from the point of view of their thermodynamics. The calculations and UV/VIS spectroscopic analysis of OA showed that the enol form of the compound is available in ethanol, water and diethylether. The higher stability of the OA enol form (as compared to the ketoform) was also reconfirmed by its experimental IR spectrum. Very high energy barrier of the enolization reaction of OA was calculated.

K e y w o r d s: acylaton, B3LYP functional, Krebs cycle, mechanism, oxaloacetate, theoretical study.

INTRODUCTION

The first step of the citric acid cycle (Krebs cycle) is the condensation of acetyl-CoA with oxaloacetate (oxaloacetic acid, OA) to form citrate (citric acid, CA) [1]. This reaction is catalyzed by the enzyme citrate synthase (EC 1.1.1.37). During the reaction the methyl carbon atom of the acetyl group is joined to the carbonyl group of the oxaloacetate. As a result, citroyl—CoA is formed in the active site of the enzyme as a transient intermediate [1]. The mechanism of this reaction is described in the famous book of Lehninger [1]. It is illustrated in Fig. 1. The second step of the mechanism involves an enolization of acetyl—CoA to an enol intermediate. However, regarding the stability and electron structure of oxaloacetate and acetyl—CoA, it should be expected that the latter is enolized easier than OA. In the present paper, acylated cysteamine (AC) is used as a fragment from the acetyl—CoA skeleton (cysteamine is the final compound of CoA).

Karpusas et al. [2] have studied the crystal structure of the ternary complex synthase—oxaloacetate—carboxymethyl—CoA to a resolution of 1.9 Å and refined to a conventional crystallographic *R* factor of 0.185. They have found that the condensation reaction proceeds through a neutral enol rather than an enol intermediate [2].

It is known that the conformations of biomolecules, especially those in the active sites of enzymes, have configurations quite distinguished from the most stable configuration in the isolated state. For example, Mulholland et al. [3] have found at the MP2/6-31+G(*d*) and RHF/6-31+G(*d*) levels that the OA conformation bound to the citrate synthase has higher energy than the most stable conformation, found with the approximation of isolated molecules (corresponding to a minimum). Moreover, a comparison with crystallographic structures of CA has shown that CA synthase enforces the so-

* E-mail: vdelchev@pu.acad.bg

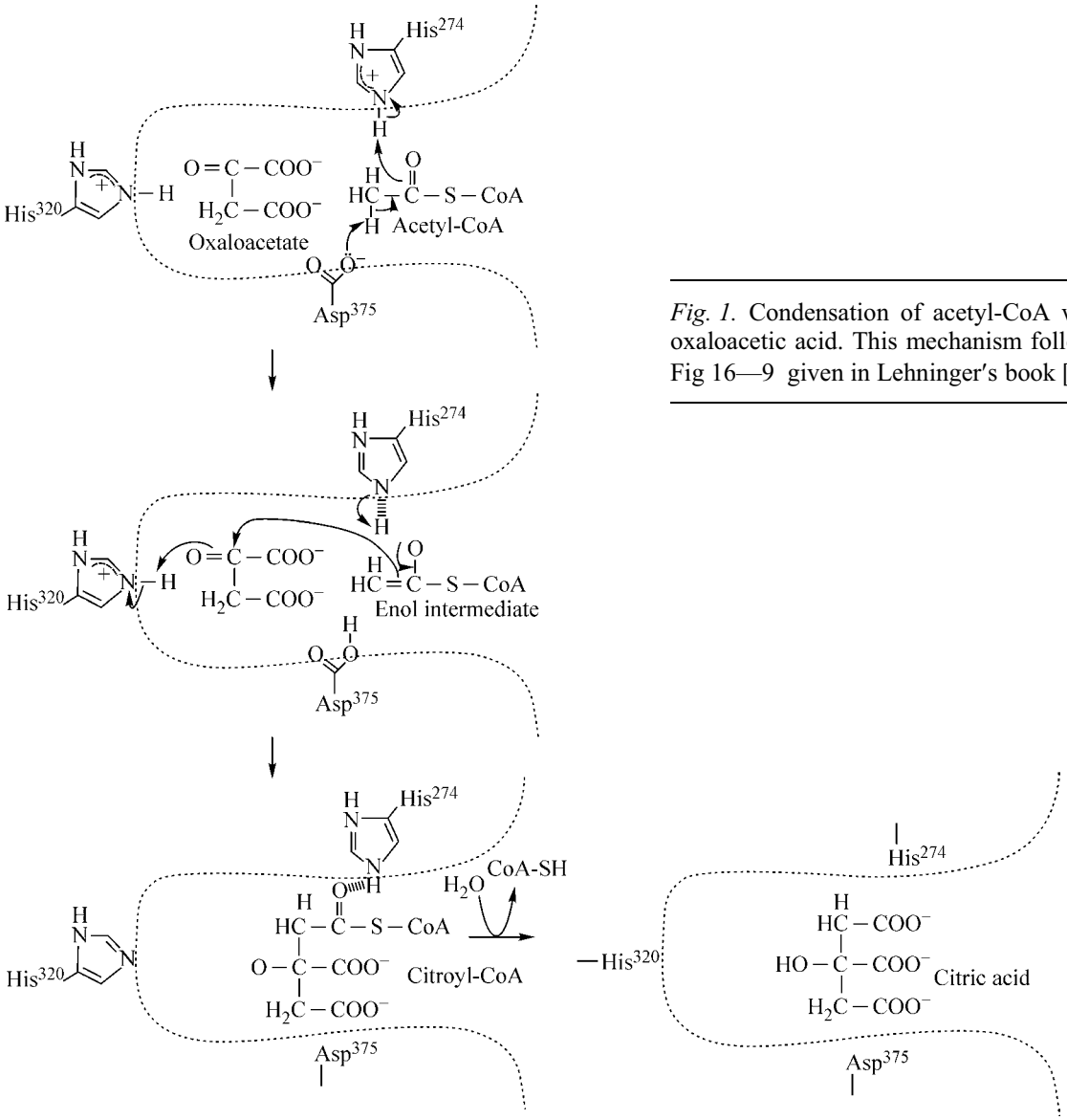


Fig. 1. Condensation of acetyl-CoA with oxaloacetic acid. This mechanism follows Fig 16—9 given in Lehninger′s book [1]

called ″citrate-like″ conformation on OA at the active site [3]. This structure can facilitate in a greater extent the Claisen reaction with acetyl—CoA. The mechanism and energy barriers of geometrical changes in OA have been discussed in [3]. Unfortunately, no data can be found on the ability of OA to enolize. Tayyari et al. [4] have found that the enol proton in dimethyl—OA moves in an asymmetric single minimum potential with a hydrogen bond strength of 31.1 kJ mol^{-1}, 35.3 kJ mol^{-1}.

The bonding of OA in the active site of citrate synthase has been described well [2]. There are several recently published works treating the substrates of this enzyme at different theoretical levels [2, 5]. Milholland et al. [2] have studied the mechanism of the OA acylation, however following the scheme proposed by Lehninger [1], i.e. the mechanism described in Fig. 1.

The purpose of this paper is to study all possible mechanisms of OA acylation (condensation) with AC (using the approximation of isolated molecules) with respect to their thermodynamics. The calculations are performed at the B3LYP [6] level of theory. The DFT hybrid functional B3LYP has shown good accuracy for studying such processes [7—10] with comparatively low cost in computation time [11]. It has been reported that the hybrid functional B3LYP yields fair agreement with experimental parameters of flexible molecules [12—17]. Furthermore, the B3LYP optimized geometries and energies of such flexible (adrenaline and noradrenaline) molecules are very similar to those found with the more expensive MP2 method [18].

COMPUTATIONAL DETAILS

The most stable conformation (in the approximation of isolated molecules) of the studied here molecular systems were found using a molecular mechanics force field. Subsequent optimizations were carried out with the GAUSSIAN-98 program package [19] at the B3LYP [6] theoretical level. The basis set 6-311G(*d*,*p*) was used with inclusion of one (+) and two (++) diffuse functions. Frequency calculations were performed to prove that the optimized structures correspond to minima on the full coordinate hyperspace. The lack of imaginary frequencies in the vibration spectra of the molecular systems proved that they lie in such minima.

The transition states of the intramolecular hydrogen transfers were investigated with the QST2 approach implemented in the GAUSSIAN-98 program [19]. The presence of one imaginary frequency in the vibration spectrum of each transition state determines it as a first order saddle point on the energy hypersurface.

The excited state calculations of the OA tautomeric forms were carried out with the CIS(50-50, Nstates=3) approach implemented in the GAUSSIAN-98 program [19].

EXPERIMENTAL

The IR spectrum of OA (SIGMA) was recorded in a KBr (SPECTRANAL) disc on a Perkin-Elmer IR/FT Spectrophotometer 1750. The UV spectra of OA were recorded on a Perkin-Elmer Lambda 15 UV/VIS Spectrophotometer in solvents ethanol, water and diethyl ether.

RESULTS AND DISCUSSION

The B3LYP/6-311++G(*d*,*p*) optimized structures of the two tautomeric forms of OA, CA, AC (keto and enol) and cysteamine are depicted in Fig. 2. The most stable tautomer of OA is OA—K. In its structure two intramolecular H-bonds are available: H(9)…O(10) and H(1)…O(13). The existence of an intramolecular H-bonds can also be seen from the experimental IR spectrum of oxaloacetic acid (SIGMA) recorded in a KBr disc. For example, the carboxylic C=O stretchings are detected at 1642 and 1691 cm^{-1}, and ν(OH) of the COOH groups are observed at 3119 cm^{-1}. All these bands are down-

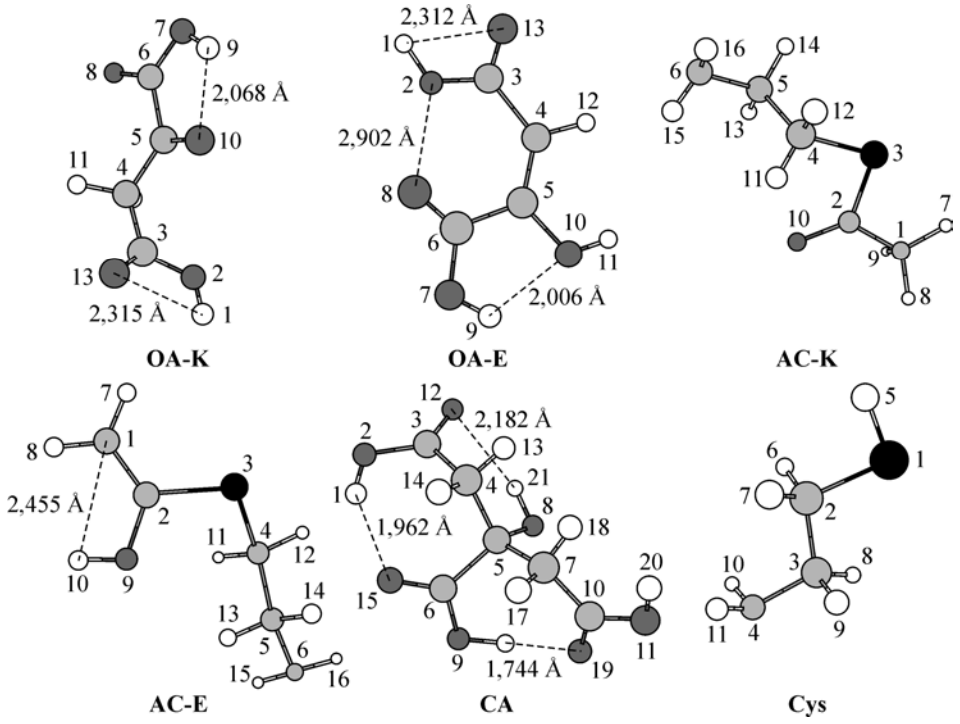


Fig. 2. Optimized geometries of the studied compounds at the B3LYP/6-311++G(*d*,*p*) level

Table 1

UV bands of the enol and ketoform of OA (B3LYP/6-311++G(*d*,*p*))

State	Ketoform			Enol form			
	λ, nm	*E*, eV	*f* *	λ, nm	*E*, eV	*f* *	Exp.
S_3	367	3.375	0.0000	271	4.568	0.0026	304[a]
S_2	274	4.517	0.0004	260	4.761	0.0077	253[b]
S_1	238	5.211	0.0000	227	5.463	0.0217	238[a], 238[c]

* Oscillator strength. [a] Diethyl ether; [b] ethanol; [c] water.

shifted as in molecular systems forming H-bonds. Most of the bands of the ketoform and enol form are overlapped and it is difficult to assign each of the observed bands to a certain tautomeric form. However, the low values of the characteristic C=O stretching vibrations show that the carboxylic C=O groups are conjugated, which is possible only in the enol form. Moreover, the band at 1111 cm^{-1} could be assigned to the C—OH stretching vibration in the enol form. The broad band at 3119 cm^{-1} involves, probably, all OH stretchings of the carboxylic group and enol OH. The experimental band at 1304 cm^{-1} appears only for enols in *trans*-configuration.

In order to check the stability of the two tautomeric forms of OA in solvents (ethanol, water, and diethyl ether), their electron transition energies were calculated at the B3LYP/6-311++G(*d*,*p*) level. The first three singlet excited states were studied. The resulted energies and electron transition probabilities (oscillator strength) data are listed in Table 1. According to Table 1, only the singlet excited states of the enol form of OA are possible. Their bands are observed in the UV/VIS spectrum of OA in ethanol, water and diethyl ether. Therefore, in these solvents the enol form should be available since these tautomers in a greater extent can form intermolecular H-bonds with the solvent molecules, especially with protic solvents like water and ethanol.

The experimental vibration frequencies of CA have been commented in ref. [20].

It was established that the inclusion of diffuse functions of hydrogens (++) and the diffuse functions of heavy atoms (+) in the basis set does not change significantly the calculated frequencies. In most cases, the values are equal. Correlating the calculated and experimental frequencies in the interval 1400—3500 cm^{-1}, we found very good ($R^2 > 0.9$) linear dependencies. Their angular coefficients $\nu_{exp} = f(\nu_{theor})$ are: B3LYP/6-311G(*d*,*p*): 0.91; B3LYP/6-311+G(*d*,*p*): 0.90; B3LYP/6-311++G(*d*,*p*): 0.90.

In the calculated enol form of OA (OA—E, conformationally corresponding to the ketoform) there are two intramolecular H-bonds as well: one within the carboxyl group and one between the enol oxygen and the carboxyl hydrogen. Despite the close distance between O(8) and O(2), this enol form seems to be rather stable.

Acetylcysteamine (AC) can also have two tautomeric forms: a ketoform (AC—K) and enol form (AC—E). Their optimized structures with the combination B3LYP/6-31++G(*d*,*p*) are depicted in Fig. 2. The enol hydrogen in the AC—E form is conducted to the π-electron density between the C(1) and C(2). This effect and the partial rehybridization of the sulfur atom leads to a planar configuration of the residue HO(9)—C(2)S(3)—C(1)H(8)H(7).

Keto-enol transformations in OA and AC. In order to clarify better the mechanism of acylation of OA to citric acid, we investigated the intramolecular keto-enol conversions in OA and AC. The optimized structures of the transition states are depicted in Fig. 3. The transition state of the transformation OA—E ⇄ OA—K is a structure situated between the two minima of the tautomeric forms. The parallel mode of the transition state (at –2193 cm^{-1}) describes the motion of H(11) between O(10) and C(4). In other words, this motion clearly depicts the dynamics of the conversion along the reaction pathway. The energy diagram of the keto-enol transformation is shown in Fig. 4, *a*. The relative energy (E_{rel}) of the molecular structures was calculated with respect to the most stable form (in both cases this is the ketoform, whose energy was taken as a conventional zero). Fig. 4, *a* clearly shows that

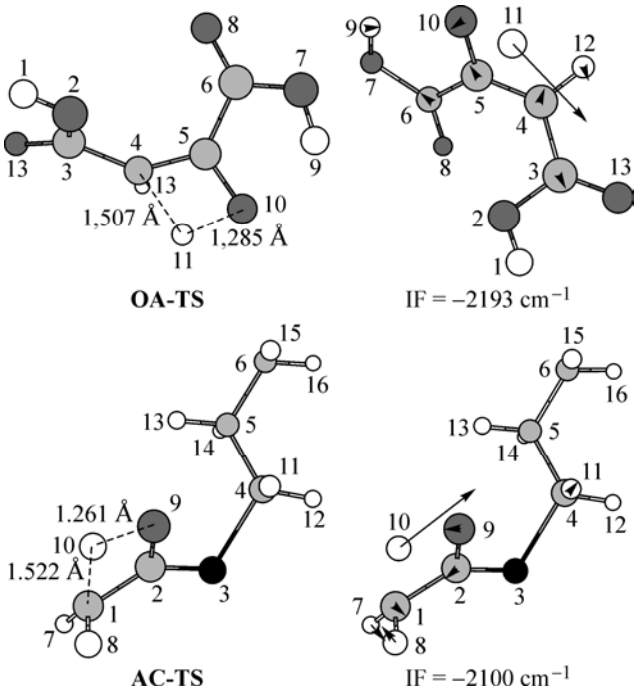


Fig. 3. Calculated structures (B3LYP/6-311++G(*d*,*p*)) of the transition states of the keto-enol transitions in OA and AC. IF — imaginary frequency

the enolization process is thermodynamically disfavoured. It occurs with an energy barrier of 283 kJ mol^{-1}, in the approximation of isolated molecules. The ketonization process has an energy barrier of 231 kJ mol^{-1}. It is accompanied with heat liberation and a positive steric factor ($T\Delta S^0_{298}$ = 2 kJ mol^{-1}). Despite the presence of intramolecular H-bonds in the OA—K tautomer, the ketonization has positive entropy change.

The transition state of the keto-enol conversion AC-E ⇄ AC-K is given in Fig. 3. In its vibration spectrum, one imaginary frequency (at –2100 cm^{-1}) corresponding to a parallel mode was calculated. In the approximation of isolated molecules, the energy diagram in Fig. 4, *b* illustrates that the enolization of AC is much more disfavoured than the enolization of OA. Therefore, the enolization of OA should occur easier and this may change the mechanism of OA acylation. In other words, the proposed mechanism in the active site of citrate synthase [1] could not explain the acylation of OA in the approximation of isolated molecules. It should be expected that the mechanism of the OA acylation is to pass through the enol form of OA rather than AC—E.

The calculated with all combinations energy barriers and thermodynamic parameters of the OA and AC keto-enol conversions are given in Table 2. At one theoretical level and different numbers of diffuse functions in the basis set, the values of the energy parameters are almost the same. Therefore, they are not sensitive to the inclusion of diffuse functions of heavy atoms or hydrogens. Furthermore, some of the values are equal. At the B3LYP level, the difference between the predicted energies of the transition states with diffuse functions of heavy atoms (+) and included diffuse functions of hydrogens (++) is less than 1 kJ mol^{-1}. However, the same difference between basis sets 6-311G(*d*,*p*) and 6-311+G(*d*,*p*) is more than 25 kJ mol^{-1}. Obviously, the initial inclusion of diffuse functions in the basis set 6-311G(*d*,*p*) influences the calculated energies of the molecular systems.

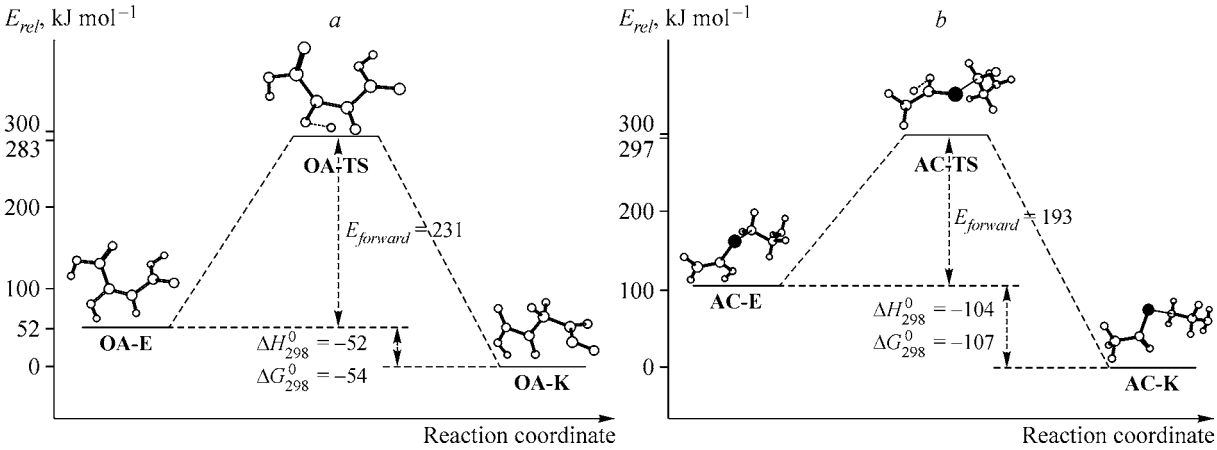


Fig. 4. Energy diagrams of the keto-enol conversions in *a*) OA, and *b*) AC. All values are in kJ mol^{-1}

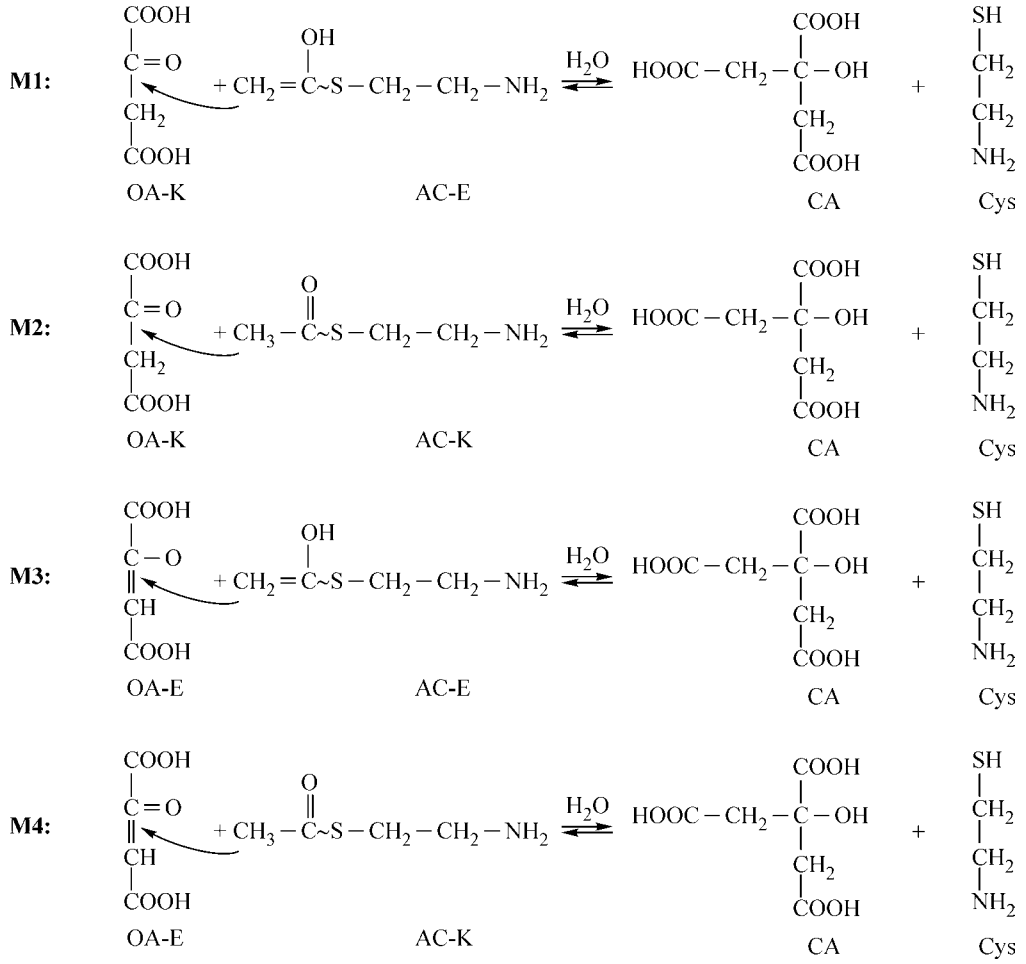
Table 2

Energy barriers and thermodynamic parameters of the keto-enol conversions of OA and AC

Method / Basis set	OA—E ⇄ OA—K					AC—E ⇄ AC—K				
	E_{TS}	E_{forw}	E_{rev}	ΔH^0_{298}	ΔG^0_{298}	E_{TS}	E_{forw}	E_{rev}	ΔH^0_{298}	ΔG^0_{298}
B3LYP/6-311G(*d*,*p*)	–531.014311	228	283	–55	–57	–686.010968	192	298	–105	–108
B3LYP/6-311+G(*d*,*p*)	–531.031361	231	283	–52	–54	–686.020948	193	297	–103	–107
B3LYP/6-311++G(*d*,*p*)	–531.031506	231	283	–52	–54	–686.021196	193	297	–104	–107

E_{TS} — energy of the transition state (a.u.); E_{forw} and E_{rev} — energy barrier of the forward and reverse conversions (kJ mol^{-1}); ΔH^0_{298} and ΔG^0_{298} in kJ mol^{-1}.

Mechanisms of OA acylation. Four possible mechanisms of OA acylation in the approximation of isolated molecules (298 K, 1 atm) could be proposed. They are illustrated in Scheme 1.



Scheme 1. Suggested mechanisms of the OA acylation

All mechanisms described in Scheme 1 are theoretically possible. They have equal probability to occur because the electron density at C(1) in AC—E and AC—K is large and, therefore, they both could act as nucleophilic agents attacking the C(5) atom in the OA—E or OA—K.

Table 3

Thermodynamic parameters (kJ mol^{-1}) *of the OA acylation mechanisms*

Method / Basis set	M1			M2			M3			M4		
	ΔH^0_{298}	ΔG^0_{298}	$T\Delta S^0_{298}$	ΔH^0_{298}	ΔG^0_{298}	$T\Delta S^0_{298}$	ΔH^0_{298}	ΔG^0_{298}	$T\Delta S^0_{298}$	ΔH^0_{298}	ΔG^0_{298}	$T\Delta S^0_{298}$
B3LYP/6-311G(*d*,*p*)	–127	–70	–57	–22	38	–60	–182	–127	–55	–77	–19	–58
B3LYP/6-311+G(*d*,*p*)	–96	–40	–56	7	67	–60	–148	–94	–54	–45	13	–58
B3LYP/6-311++G(*d*,*p*)	–96	–40	–56	6	67	–61	–148	–94	–54	–46	13	–58

The mechanism M1 has been described in Lehninger′s book [1] as the most realistic one occurring in the active site of the enzyme citrate synthase. In order to check if this is true in the approximation of isolated molecules, we investigated the thermodynamics of mechanisms in Scheme 1, at all theoretical levels. The data are listed in Table 3. The estimated values clearly show that the mechanism M2 is thermodynamically disfavoured. Thus, an interaction between the ketoforms of OA and AC could not occur. The most probable mechanism is the M3 one. It occurs between the enol forms of OA and AC and it has the largest negative value of ΔG^0_{298}. Moreover, this mechanism is accompanied with insignificant steric changes in the reactant geometries (low steric factor $T\Delta S^0_{298}$). At the second position the most probable mechanism is the M1 one. With respect to the stability of the enol forms of OA and AC, we should conclude that the simultaneous enolization of OA and AC could happen with a low probability. Moreover, as it was mentioned above, the enolizations of OA and AC pass through very high energy barriers. In this way, the mechanism M1 could be determined as a more realistic one, in the approximation of isolated molecules, than the mechanism M3. However, in the enzyme′s active site, the mechanism M3 can occur easier than the M1 one. For example, at the first step both OA and AC enol forms could arise simultaneously, due to the interaction of O(10) with the His^{320} hydrogen (Fig. 1). This interaction leads to an intermolecular H exchange between oxaloacetate and His^{320} (for several organic compounds it has been proven that the inermolecular H exchange between tautomeric forms has more than five times lower energy barrier than the intramolecular one [21—24]). The next steps follow those given in Fig. 1. Obviously, both mechanisms in the active site of the enzyme (M1 and M3) can be treated as alternatives to one another.

The data from Table 3 show also that all mechanisms are enthalpically favoured, however the largest heat effect was calculated for the M3 mechanism.

CONCLUSIONS

In order to follow the mechanism of the acylation of oxaloacetic acid with acetyl—CoA, we performed theoretical calculations at the B3LYP level using 6-311G(*d*,*p*) basis set with different numbers of diffuse functions (without, +, and ++). The results led to the next interesting conclusions:

1. Comparing theoretical and experimental (KBr disc) IR bands of OA, it was established that the enol form is available in the crystal state. In solvents like ethanol, water and diethyl ether the calculated and experimental UV/VIS bands show abundance of the enol form of OA as well.

2. In the approximation of isolated molecules, the enol form of OA has higher energy than the ketoform; moreover, the transition OA—K $\rightleftarrows$ OA—E is thermodynamically disfavoured. However, the enolization of OA can occur in solvents (e.g. water, which is available in the living organisms), especially protic solvents, which could assist in the proton transfer in OA.

3. The calculations showed that the enol form of AC easily converts into the ketoform as the latter is the most stable tautomer. Therefore, ketoform can participate directly in the acylation of OA in the active site of the enzyme citrate synthase.

4. The mechanism of acylation of OA having the largest negative value of ΔG^0_{298} is M3 one. It suggests an acylation between the enol forms of OA and AC. However, since the enolizations of OA and AC pass through high energy barriers, this process could be determined as forbidden. We believe

that it could happen in the active site of the enzyme where the enolization processes in OA an AC are assisted by the molecules of water, His^{274}, Asp^{375}, and His^{320} (intermolecular H-transfers).

Acknowledgements. One of us (*VBD*) thanks the National Science Fund (Ministry of Education and Science of Bulgaria) for the financial support (Project ″VUH-17/05″). We thank also Bistra Stambolyiska (Institute of Organic chemistry, Bulgarian Academy of Sciences) for performing the calculations with Gaussian-98 program.

REFERENCES

1. *Lehninger A.* Principles of Biochemistry., 4 ed. – N4.: Freeman, 2004. – P. 608.
2. *Karpusas M., Branchaud B., Remington S.J.* // Biochem. – 1990. – **29**. – P. 2213 – 2219.
3. *Mulholland A.J., Richards W.G.* // J. Mol. Struct. (THEOCHEM). – 1998. – **429**. – P. 13 – 21.
4. *Tayyari S.F., Salemi S., Tabrizi M.Z., Behforouz M.* // J. Mol. Struct. – 2004. – **694**. – P. 91 – 104.
5. *Mulholland A.J., Richards W.G.* // J. Mol. Struct. (THEOCHEM). – 1998. – **427**. – P. 175 – 184.
6. *Becke A.D.* // J. Chem. Phys. – 1993. – **98**. – P. 5648 – 5652.
7. *Mons M., Dimicoli I., Piuzzi F.* // Int. Rev. Phys. Chem. – 2002. – **21**. – P. 101 – 135.
8. *Sevilla M.D., Becker D., Yan M., Summerfield S.R.* // J. Phys. Chem. – 1991. – **95**. – P. 3409 – 3415.
9. *Hendricks J.H., Lyapustina S.A., de Clercq H.L., Bowen K.H.* // Ibid. – 1998. – **108**. – P. 8 – 11.
10. *Orlov V.M., Smirnow A.N., Varshavsky Y.M.* // Tetrahedron Lett. – 1976. – **17**. – P. 4377 – 4378.
11. *Li X., Cai Z., Sevilla M.D.* // J. Phys. Chem. B. – 2001. – **105**. – P. 10115 – 10123.
12. *Carney J.R., Zwier T.S.* // J. Phys. Chem. A. – 2000. – **104**. – P. 8677 – 8688.
13. *Carney J.R., Zwier T.S.* // Chem. Phys. Lett. – 2001. – **341**. – P. 77 – 85.
14. *Graham R.J., Kroemer R.T., Mons M. et al.* // J. Phys. Chem. A. – 1999. – **103**. – P. 9706 – 9711.
15. *Snoek L.C., Robertson E.G., Kroemer R.T., Simons J.P.* // Chem. Phys. Lett. – 2000. – **321**. – P. 49 – 56.
16. *Butz P., Kroemer R.T., MacLeod N.A., Robertson E.G., Simons J.P.* // J. Phys. Chem. A. – 2001. – **105**. – P. 1050 – 1056.
17. *Butz P., Kroemer R.T., .MacLeod N.A., Simons J.P.* // Ibid. – 2001. – **105**. – P. 544 – 551.
18. *van Mourik T., Emson L.E.* // Phys. Chem. Chem. Phys. – 2002. – **4**. – P. 5863 – 5871.
19. *Frisch M.J., Trucks G.W., Schlegel H.B., Scuseria G.E., Robb M.A., Cheeseman J.R., Zakrzewski V.G., Montgomery J.A. Jr., Stratmann R.E., Burant J.C., Dapprich S., Millam J.M., Daniels A.D., Kudin K.N., Strain M.C., Farkas O., Tomasi J., Barone V., Cossi M., Cammi R., Mennucci B., Pomelli C., Adamo C., Clifford S., Ochterski J., Petersson J.A., Ayala P.Y., Cui Q., Morokuma K., Malick D.K., Rabuck A.D., Raghavachari K., Foresman J.B., Cioslowski J., Ortiz J.V., Stefanov B.B., Liu G., Liashenko A., Piskorz P., Komaromi I., Gomperts R., Martin R.L., Fox D.J., Keith T., Al-Laham M.A., Peng C.Y., Nanayakkara A., Gonzalez G., Challacombe M., Gill P.M.W., Johnson B., Chen W., Wong M.W., Andres J.L., Gonzalez C., Head-Gordon M., Replogle E.S., Pople J.A.* Gaussian-98., A.3. 1998., Revision., Gaussian Inc.., Pittsburgh PA.
20. SDBSWeb: http://www.aist.go.jp/RIODB/SDBS/ (National Institute of Advanced Industrial Science and Technology).
21. *Gorb L., Leszczynski J.* // J. Amer. Chem. Soc. – 1998. – **120**. – P. 5024 – 5032.
22. *Podolyan Y., Gorb L., Leszczynski J.* // Int. J. Mol. Sci. – 2003. – **4**. – P. 410 – 421.
23. *Chandra A.K., Nguyen M.T., Uchimaru T., Zeegers-Huyskens T.* // J. Phys. Chem. A. – 1999. – **103**. – P. 8853 – 8860.
24. *Gu J., Leszczynski J.* // Ibid. – 1999. – **103**. – P. 2744 – 2750.