Eur Biophys J (1993) 22:207–212

European Biophysics Journal

AM1 and PM3 study of a low molecular weight structural mimic of hydrogen exchange within the catalytic center of aspartic proteases

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Received: 4 December 1992 / Accepted in revised form: 1 April 1993

Abstract. Based on recent X-ray studies, a low molecular weight model of the active center of aspartic proteases is proposed. The model is small enough to enable unattended geometry optimizations (including search for saddle-points) by molecular orbital methods. It consists of two malonic acid molecules and a water molecule; there is a carboxylic dimer at one end and the water molecule is located between the carboxylate and the carboxyl group at the other. The latter structure reproduces the geometry of the catalytic center of the native enzyme penicillopepsin with a root-mean-square deviation of 0.46 Å for five O--O distances. The AM1 and PM3 molecular orbital methods were used to study the H-bond exchange within the model. Both methods lead consistently to the following conclusions: Among 2 pairs of symmetry-equivalent stationary states of the catalytic center there are at least 4 symmetry-independent hydrogen-exchange pathways, and many more when including symmetry of the center. Energetics and geometry of all identified pathways are presented. In summary, they result in "juggling" all three active center protons (COOH and HOH) among all five active center oxygens (COO^-, COOH and H_2O) providing the center with a high delocalisation with respect to the actual position of its anionic site and/or its protonation status. The relevance of the delocalisation of the acidic proton to the mechanism of enzymatic action is briefly discussed.

Key words: AM1-PM3-MOPAC-aspartic – Proteases-catalytic – Center – Mimic-hydrogen exchange

Introduction

The aspartic proteases are a family of enzymes characterized by having their catalytic centers formed (typically as monoions) from two aspartic acid side chain terminii placed nearly coplanar in a C_2 locally symmetrical environment, Fig. 1 a (see recent review by Davies 1990). They include the monomeric two-domain enzymes (pepsin, renin, chymosin, and the fungal enzymes), as well as the homodimeric retroviral enzymes [HIV-1 protease (Navia et al. 1989; Wlodawer et al. 1989; Lapatto et al. 1989) and Rous sarcoma virus protease (Jaskólski et al. 1990)]. Once the essential role of the HIV-1 protease to the retroviral life cycle was realised (Kohl et al. 1988), studies aimed at the elucidation of the mechanisms of catalysis and inhibition of aspartic proteases received an enourmous impetus as a significant target in fighting AIDS. Selected late examples include: (Hyland et al. 1991 a, b; Jaskólski et al. 1991; Thaisrivongs et al. 1991; Kuzmič et al. 1991; James et al. 1992; Fraser et al. 1992; Griffiths et al. 1992; Dreyer et al. 1992; Furfine et al. 1992; Parris et al. 1992) and references therein. Very recently, a concensus has been reached as to the molecular mechanism of catalysis. On the one hand, the mechanism has been deduced from X-ray studies on complexes of fungal enzymes (penicillopepsin, endothiapepsin and rhizopuspepsin) with *gem*-diol type inhibitors (James et al. 1992; Veerapandian et al. 1992; Parris et al. 1992, respectively) most accurately mimicking the tetrahedral intermediate implied during the catalytic hydrolysis of a peptide bond (James and Sielecki 1985). On the other hand, a similar mechanism has been implied from equilibrium/kinetics studies on the interaction of the HIV-1 protease with inhibitors (Hyland et al. 1991 a, b). Thus, the catalytic mechanism appears to be common for both retroviral and cell proteases and resembles that previously proposed by Suguna et al. (1987).

The only tool capable of providing meaningful results on geometries and energies of transition states of providing meaningful results on geometries and energies of transition states involved in reaction mechanisms is state-of-the-art quantum mechanics. Obviously, a direct use of molecular orbital methods is precluded for systems of more than ca. 100 atoms in general, and for enzyme-substrate complexes in particular. Hence, there is a need for low molecular weight models which, even if simplistic,

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could still reliably predict major aspects of the mechanisms in question. An earlier theoretical study for the model $HCOO^-$...HOH...HCOOH system constrained to fit the geometry of the catalytic Asp diad in the rhisopuspepsin (Suguna et al. 1987) concluded that the semiempirical "PM3 method ... could reproduce the geometry and energetics of the complex, obtained by sophisticated ab initio calculations, with astonishing accuracy" (Turi and Náray-Szabó 1992). Two other theoretical works, one by semiempirical MNDO (Goldblum 1988) and the second by the ab initio method, 6-31G* basis set, (Rao and Singh 1991) dealt with the protonation status and acidity within the Asp diad. The latter work implied that the molecular mechanics/dynamics used [i.e. AMBER version 3.3 (Singh et al. 1986)] could not reproduce, *with no extra constraints,* a nearly coplanar arrangements of the COO^-/COOH diad, a landmark feature of the active centers in all aspartic proteases (Davies 1990). The work also indicated a significant variation (1–16 kcal/mol) of the energy barrier to the hydrogen exchange between "the inner" oxygens (Fig. 1), depending on whether the active center carboxyl and carboxylate were or were not constrained to the geometry found in the reference X-ray structure (Suguna et al. 1987).

All three theoretical works used selected X-ray structures and *assumed* that the undissociated hydrogen would protonate one of the inner oxygens in the catalytic Asp diad, in contrast with the novel mechanistic hypothesis, also based on X-ray studies, which *assumed* an outer oxygen to be protonated at the reaction starting point (James et al. 1992; Parris et al. 1992; Hyland et al. 1991 a, b). Clearly, an X-ray study of a macromolecule is usually incapable of localizing hydrogens since the latter are almost transparent to X-rays. However, the assumptions should not be considered as contradictory since there is more than enough evidence that acidic hydrogens, i.e. hydrogens involved in H-bonds, are not fixed within a protein structure but are subject to a continuous exchange between (among) the respective hydrogen bond partners (Petsko and Ringe 1984); in fact, they are disordered.

The purpose of this work is twofold: (*i*) Using molecular mechanics we are going to demonstrate that the acidic proton does not have a clearly defined preference for any to the four carboxyl oxygens within the catalytic diad, and (*ii*) applying quantum mechanics to a simplistic, yet reasonably fitting, model we are going to prove that extensive hydrogen exchange should operate in the catalytic center and be compatible with a momentary protonation of any of the four carboxyl oxygens of the catalytic diad.

The AM1 (Dewar et al. 1985) and PM3 (Stewart 1989) semiempirical molecular orbital methods were chosen since they are believed to be the present state-of-the-art for semiempirical molecular orbital methods. With this choice we hoped to reach a level for the treatment consistent with the sizes of our models, which even if not critical in this work, would become prohibitively large for use of more accurate high-level ab initio methods in related projects currently in progress. Despite some controversy over whether AM1 and PM3 differ in a significant way (Dewar et al. 1990; Stewart 1990), and a recent report from the Kollman group (Schröder et al. 1991) on the superiority of PM3 over AM1, there are numerous reports of the succesful treatment of hydrogen bonds by AM1 (Marcos et al. 1988; Dannenberg 1988; Galera et al. 1988). Thus, we decided to use both the AM1 and PM3 methods in parallel and compare their operation afterwards.

Methods

The calculations reported here were carried out on an IBM-compatible PC/i486/33 MHz/16 MB computer using the AM1 (Dewar et al. 1985) and PM3 (Stewart 1989) molecular orbital models as implemented within the MOPAC program, version 6.00 (Heimer et al. 1991), which utilizes the protected mode of the i486 processor. Encounter complexes, reaction paths and transition states were analysed by standard tools available within MOPAC. Specifically, energy minima were refined with the PRECISE and/or EF (Eigenvector Following) options, while saddlepoints were found using procedures PATH and/or SADDLE (Dewar et al. 1984) followed by gradient minimization with the NLLSQ or TS procedure. Subsequently, the FORCE routine was used to check if the structure detected had only one negative normal frequency mode, indicative of a true saddlepoint. Chemical structures were edited and molecular images were prepared using the commerical PCMODEL and PCDISPLAY programs (Gajewski and Gilbert 1991). Initial optimizations of any encounter complexes were done by molecular mechanics MMX (Gajewski et al. 1990) as implemented within the PCMODEL program.

A substructure comprising the enzyme's active center and its nearest surroundings was built for reference and for studying the most probable site of protonation within the $COOH/COO^-$ catalytic diad. The X-ray coordinates of penicillopepsin (James and Sielecki 1983) were used. The model was composed of the sequence fragments -Asp^{33}-Thr^{34}-Gly^{35}-Ser^{36}- and -Asp^{213}-Thr^{214}-Gly^{215}-Thr^{216}- plus the water molecule centrally located over the carboxyl/carboxylate diad. Both fragments were capped at their *N*-termini by acetyl groups (overlapping the $C^{\alpha}-C=O$ coordinates of the Phe^{32} and Ala^{212} residues, respectively) and included the carbonyl carbons of Ser^{36} and Thr^{216} as the last atoms on their *C*-terminal sides. The position of the acidic hydrogen was systematically varied among the outer and the inner oxygen atoms of the catalytic diad's Asp^{33} or Asp^{213} carboxyl/carboxylate groups and the system was energy-minimized for each specific configuration, using either the united atom or all atom MMX force field (Gajewski et al. 1990).

Results and discussion

The size of protonation

The set of optimized reference models described in the Methods section was fitted to the enzyme's X-ray geom-

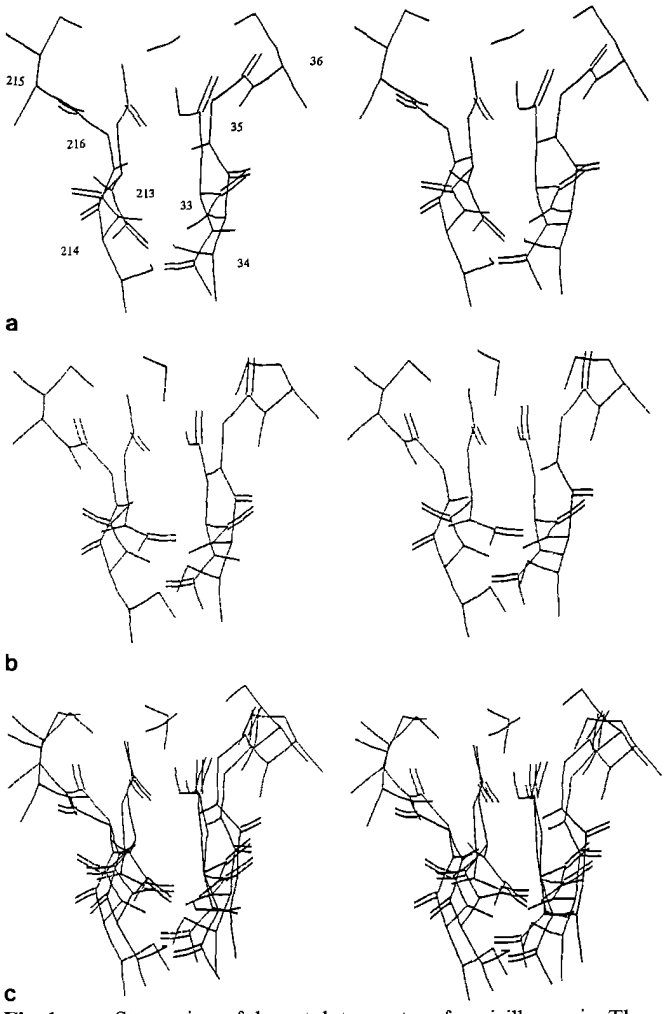

Fig. 1 a–c. Stereoview of the catalytic center of penicillopepsin. The model was composed of the sequence fragments -Asp[33]-Thr[34]-Gly[35]-Ser[36]- and -Asp[213]-Thr[214]-Gly[215]-Thr[216]-; the starting geometry taken from the x-ray of penicillopepsin (James and Sielecki 1983). The numbers of amino acid residues are indicated **a** *solid line*; energy-refined structure **b** *dashed line*; energy-refined structure superposed on the x-ray structure **c**. The refinement was done by the MMX/united atom method, with the Asp^{33} inner oxygen protonated. It is seen that only some peripheral atoms in the selected fragment do not overlap tightly after the refinement, making the rms = 0.37. The catalytic diad has the same structure in both cell and retroviral proteases: Two sequence fragments -Asp-Thr-Gly-Thr- are locked into two β-turns CO(Asp)...HN(Thr) and crosslinked by two sets of inter-sequential hydrogen bonds from NH(Thr) of one sequence to O^{γ}(Thr) of the neighbouring sequence and vice versa. In addition, there are two symmetry-related pairs of intra-sequential hydrogen bonds apparently maintaining the active center COO^- and COOH groups coplanar. These are the NH(Gly) to the inner oxygens of COO^- and/or COOH bonds and the OH^{γ}(Thr) to the outer oxygens of COO^- and/or COOH bonds. There is no concensus as to wether the inner oxygens are hydrogen cross-linked with each other or not. One of the two proximal Asp carboxyls is always dissociated and in the free enzyme at least one water molecule is permantently captured between and somewhat above the twin carboxylic groups

etry (James and Sielecki 1983). The purpose of this test was to assess the stability of the active center's reference model, the reliability of the MMX force field used, and to find the most likely site of protonation in the COOH/COO^- diad. The best fit was achieved for the model

Table 1. Structure changes, represented as the RMS values (in Å), that accompanied the MMX refinements of variously protonated active centers. The X-ray geometry of penicillopepsin was the reference. See text for the definition of the active center structure referred to in the table

Protonation site	MMX/ united atom	MMX/all atom
Asp^{33} inner	0.37	0.41
Asp^{33} outer	0.44	0.44
Asp^{213} inner	0.46	0.53
Asp^{213} outer	0.42	0.39

having the inner oxygen of Asp^{33} protonated, minimized by the united atom MMX method, see Fig. 1. This model was in agreement with theoretical works (Turi and Náray-Szabó 1992; Goldblum 1988; Rao and Singh 1991) and in agreement as to which of Asp^{33} and Asp^{213} side chains was protonated at the start of the mechanism. However, it was in disagreement with current mechanistic proposals (James et al. 1992; Parris et al. 1992; Hyland et al. 1991 a, b) that assume the outer oxygen of the Asp^{33} side chain to be protonated at the reaction start. Since other substructures, optimised either by the united atom or the all atom MMX method, also fitted the enzyme's X-ray geometry quite well, see Table 1, the question of the site of protonation seems to be open and perhaps not absolutely essential, given the well documented flexibility of acidic protons in proteins (Petsko and Ringe 1984), also confirmed by the present results. At the same time, good fits of most of the optimized substructures, and in particular a good retention of the nearly coplanar arrangement of the COOH/COO^- catalytic diad, argue in favor of the force field chosen in relation to the selected active center reference model.

The model for unattended geometry optimizations by quantum mechanics

The saddlepoint search, as implemented in MOPAC, is most conveniently done by unattended gradient optimizations over all structural variables. For this purpose, a model was sought that was simple enough to be amenable to such treatment, yet possessed all "first order" features of the catalytic diad, in particular the interacting -COOH/HOH/$^-$OOC- arranged in a geometry that would reasonably resemble that of the native enzyme. Screening a number of possibilities, we started from the simplest possible ones, such as two loose formic acid molecules plus water, or an analogous one based on two acetic acid molecules and water. Then several "bridged" models, utilizing polymethylene bridges of azelaic and sebacic acids, to keep the the terminal carboxylic groups in proximity, were analyzed. Finally, a relatively simple and highly polar model was invoked, composed of two malonic acid molecules flexibly bridged by a carboxylic acid dimer at one end, with the two other carboxylic groups and a water forming a hydrogen-bonded assembly with a reasonable resemblance to the catalytic diad at the other end of the complex.

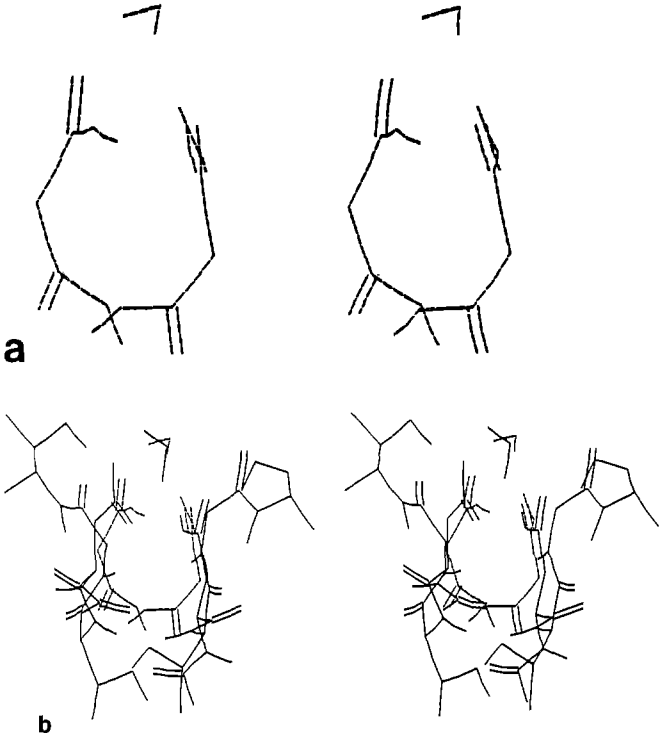

Fig. 2. Stereoview of the AM1-optimized malonic-acid dimer mimic **a** *dashed line* and the mimic superposed on the corresponding fragment of the energy-refined active center of penicillopepsin **b** The central water molecule is also included. All aliphatic hydrogens are ommited for clarity. The PM3 method gives a very similar result

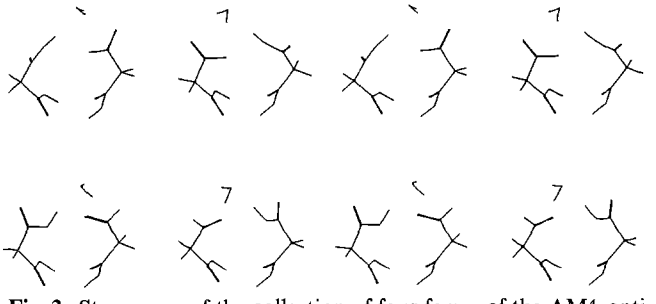

Fig. 3. Stereoview of the collection of four forms of the AM1-optimized dimalonic mimic which were used in this work as a basis for the study of a possible hydrogen exchange in the catalytic center of aspartic proteases. The four structures are marked in the text as follows: 1 *upper left*, 2 *upper right*, 3 *lower left*, 4 *lower right*. The PM3 method gives very similar results

Of all the models mentioned above, the first two appeared to be too loose. Consequently, the respective encounter complexes usually "exploded" or otherwise collapsed at some stage of AM1 and/or PM3 runs aimed at detecting saddle points; at any rate they lead to geometries incompatible with the reference in Fig. 1. It should be noticed, however, that Schröder et al. have successfully used a model at a comparative level of simplicity for studying mechanisms of action of serine proteases (Schröder et al. 1991; Dagget et al. 1991). On the other hand, the subsequent models based on covalent bridges appeared to be too stiff; that is, during similar runs they tended to get trapped within local energy minima surrounded by high barriers. Eventually, the only model that

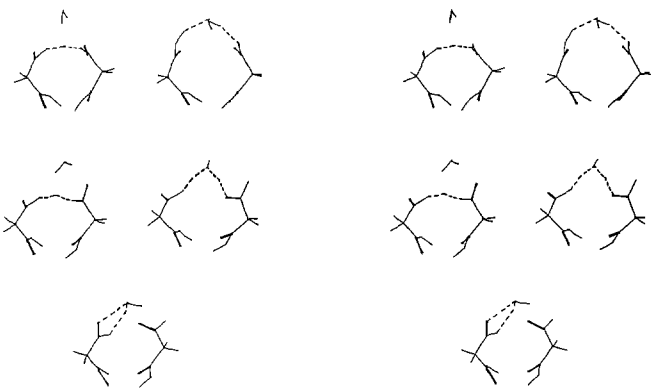

Fig. 4. Stereoview of the collection of the AM1-optimized representative transition states to the hydrogen exchange within the mimic/water complex. From *top/left to bottom/right* by rows: $1 \rightleftharpoons 2$, water mediated $1 \rightleftharpoons 2$, $1 \rightleftharpoons 4$, water mediated $1 \rightleftharpoons 4$, $1 \rightleftharpoons 3$. The O-H bonds being created/broken are represented by *dashed lines*. The PM3 method gives very similar results

passed this pre-selection was the malonic dimer with water. The "connecting end" made by the carboxylic dimer proved to be a sufficiently strong but highly flexible link. At the same time "the functional end" made of the other two carboxyls plus water reasonably and permanently met the basic geometrical requirements of their respective counterparts in the enzyme's catalytic center. Figure 2b demonstrates to what extent the optimized mimic overlaps the enzyme's active center.

The root-mean-square deviation for the five relevant O-O distances in the model, compared to either the native structure (James and Sielecki 1983) or to the MM-optimized models, see above, was invariably equal to 0.46 Å.

Hydrogen exchange within the dimalonic model

It follows from the symmetry of the dimalonic model that the hydrogen exchange should be considered and analyzed in detail among two pairs of symmetry-equivalent structures which, fully optimized by AM1, are given in Fig. 3. The respective geometries optimised by the PM3 method did not differ significantly from the former ones. It should be noticed that in the dimalonic model the pairs themselves differ only by the alternate allocations of two hydrogens within the "bridging" carboxylate dimer.

In the model given in Fig. 3, four direct carboxyl-carboxylate ($1 \rightleftharpoons 2$, $3 \rightleftharpoons 4$, $1 \rightleftharpoons 4$ and $2 \rightleftharpoons 3$) and six water mediated ($1 \rightleftharpoons 2$, $3 \rightleftharpoons 4$, $1 \rightleftharpoons 3$, $2 \rightleftharpoons 4$, $1 \rightleftharpoons 4$ and $2 \rightleftharpoons 3$) proton exchange modes can be imagined. In the direct exchange modes, reaction paths are in principle associated with the transfer of a single proton, *viz.* the carboxylic one, while in the water-mediated modes reaction paths are essentially associated with a concerted movement of the carboxylic proton to water and a water proton to an oxygen supplied either by the carboxylate or by the same carboxyl. Among them the $1 \rightleftharpoons 4$ and $2 \rightleftharpoons 3$ modes are unique while ($1 \rightleftharpoons 2$, $3 \rightleftharpoons 4$) and ($1 \rightleftharpoons 3$, $2 \rightleftharpoons 4$) make two pairs of symmetry-equivalent exchange modes.

Accordingly, three direct exchange modes and four water-mediated exchange modes were studied explicitly

Table 2. Relative AM1 energy scale[a] for the hydrogen exchange modes. Transition state energies[b] are at the intercepts of reactant rows and product colums

Reactant	Product	2	3	4
	Energy	0.1	0.1	0.0
1	0.0	11.0	–	14.0
		13.8	17.3	17.6
2	0.1		13.9	[d]
			[c]	
3	0.1			[e]

[a] AM1 heat of formation, HoF, values in kcal/mol relative to 1. Absolute HoF value for 1 equals −490.1 kcal/mol
[b] The upper row applies to direct carboxyl-carboxylate exchange while the lower row applies to water mediated exchange
[c] Saddlepoint was not indentified
[d] Equivalent to 1⇌3
[e] Equivalent to 1⇌2

Table 3. Relative PM3 energy scale[a] for the hydrogen exchange modes. Transition state energies[b] are at the intercepts of reactant rows and product columns

Reactant	Product	2	3	4
	Energy	1.8	1.8	0.0
1	0.0	16.7	–	14.8
		14.1	23.1	12.2
2	1.8		14.5	[c]
			12.0	
3	1.8			[d]

[a] PM3 heat of formation, HoF, values in kcal/mol relative to 1. Absolute HoF value for 1 equals −483.0 kcal/mol
[b] The upper row applies to direct carboxyl-carboxylate exchange while the lower row applies to water mediated exchange
[c] Equivalent to 1⇌3
[d] Equivalent to 1⇌2

to cover all the possibilities outlined above. The saddle-point energies for most of these modes of exchange were determined and optimized. They are given in Tables 2 and 3 for AM1 and PM3, respectively, together with the energies of the canonical structures from Fig. 3. Representative transition state structures are given in Fig. 4.

It is clear from the tables that most of the exchange modes studied in this work appear to be available both at the AM1 and PM3 level, and that an agreement between the two methods is quite good. The results, in fact, suffice for the conclusion that in the catalytic site model assumed in this work all three active center protons (COOH and HOH) are continually and completely "juggled" among all five active center oxygens (COO^-, COOH and H_2O), thus providing the center with a high delocalisation with respect to the actual position of its anionic site and/or its protonation status. Since the relevant distances between five oxygens mentioned above are quite similar in the present model and in the active center of aspartic proteases, we have reason to believe that similar delocalisation is also observed in the active center of a true enzyme. In particular, it seems unlikely that the energy barriers to proton exchange, ranging in our model from 12–16 kcal/mol, would be higher in an enzyme. It is noticeable that the values obtained by semiempirical methods agree well with those obtained by sophisticated ab initio methods (Rao and Singh 1991), provided that our results are compared to those in the cited work that accomodate reasonable geometrical cnostraints [compare Tables II, III and IV in Rao and Singh (1991)]. Taking into account the low barrier to proton transfer from one energy minimum to another, it can be inferred that the proton nuclear wave function will be distributed almost uniformly among the four almost equivalent minima (i.e. the proton will be highly delocalised). Therefore the C_2 symmetry of the heavy-atom frame will probably be conserved after including the proton. The agreement between the MNDO-based AM1 or PM3 and ab initio methods looks particularly attractive since it promises that the same semiempirical methods might perform successfully in studies of the catalytic mechanism itself. The latter studies, being computationally much more demanding, would be less amenable to a more rigorous ab initio treatment. Preliminary studies aimed at theoretical verification of recent mechanistic proposals (James et al. 1992; Parris et al. 1992; Hyland et al. 1991 a, b) using the dimalonic model introduced in this work, as well as its extended version, look promising (Ołdziej and Ciarkowski, unpublished results).

Acknowledgements. The financial support of this work by the Polish Scientific Research Concil (KBN) under grant No 1067/P3/92/02 is gratefully acknowledged.

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