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Density functional theory applied to copper proteins

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chapter eight

UV/VIS SPECTRA

*of
copper complexes*

CONTENTS

8.1	<i>Calibration</i>	165-168
8.2	<i>Oxidized states</i>	169-172
8.3	<i>Protein environment</i>	173-176

SUMMARY

This chapter deals with the UV/VIS spectra, or excitation energies, of copper proteins. As Time-Dependent Density Functional Theory (TD-DFT) is formally not applicable to open shell systems, use is made of the semi-empirical CNDO/1 and INDO/1 approaches. TD-DFT results for the reduced state of three copper proteins is used to calibrate the CNDO/INDO copper parameters, which are subsequently tested for the excitation energies in the oxidized state of these proteins. Also the influence of the presence of the protein on the computed excitation energies is checked.



Calibration

A direct comparison of Density Functional Theory and semi-empirical excitation energies of the reduced state of copper proteins

In recent years Density Functional Theory (DFT)¹ has been successfully applied to the computation of excitation energies and polarizabilities of (metallo-)organic molecules, by using Time-Dependent DFT (TD-DFT). It was shown to be an efficient and reliable method to compute these response properties, that compare well with experimental data. Unfortunately, TD-DFT is in principle possible only for closed-shell systems. As the most important of the two states of copper proteins (oxidized state) has an unpaired electron, and therefore is an open shell system with a doublet ground state, formally TD-DFT can not be used for the computation of the excitation energies of an active site of copper proteins in the oxidized state.

Alternatively, the excitation energies can be obtained by semi-empirical calculations using the INDO or CNDO Hamiltonian^a, which have been successfully used for the UV/VIS spectra of (metallo)organic molecules. As the excitation energies are then obtained in a Configuration Interaction calculation, no problem arises for an open shell ground state and also the oxidized state of copper proteins can be treated with it. A remaining issue that needs to be solved before one can use it straightforwardly for metalloproteins, is to check whether the standard INDO or CNDO parameters give a reasonable description for the system. Therefore the excitation energies of the reduced state of the copper proteins will be computed by using both TD-DFT and the semi-empirical approach, and the copper parameters needed in the latter will be adjusted if necessary.

An important aspect in the TD-DFT investigations was the recognition that standard exchange-correlation potentials do not give an accurate description of the energy levels of the molecular orbitals. Therefore specially designed xc-potentials^{216,299,300} have been constructed that improve the computed excitation energies (as well as other properties like the molecular polarizability²¹⁰) enormously. One of the first of these potentials is the van Leeuwen-Baerends potential (LB94)²¹⁶ that shows the correct asymptotic behavior, which is needed to obtain good polarizability values. However even though the outer region is described well, the inner region is less well described. Therefore a combination of a potential giving a good description of the inner region (Becke¹²⁰-Perdew¹²¹) and a potential giving a good description of the outer region (LB94) has been tried where the weight of each potential at a certain point is gradient regulated in order to get a smooth transition from the inner to the outer region. One additional feature of this gradient regulated asymptotically correct (GRAC)³⁰⁰ potential is that it needs the ionization potential of the molecule as input parameter. For molecules for which this property is known, one can simply use the experimental value, otherwise one has to use a computed value. In the latter case, it is recommended to use the value obtained by using the SAOP (Statistical Averaging of model Orbital Potentials) potential²⁹⁹, as this usually provides ionization potentials that compare well with experimental values.

The energy level of the Highest Occupied Molecular Orbital (HOMO) using a triple zeta valence basis set plus polarization functions (TZP) for the reduced state of the active site of

^aThe abbreviations CNDO and INDO stand for the CNDO/1 and INDO/1 Hamiltonians

three copper proteins with three exchange-correlation potentials is reported in Table 8.1.1. For *wildtype* azurin^a the orbital energy was computed with the Becke-Perdew (BP) potential, the SAOP potential and the GRAC potential (where the ionization potential obtained in the SAOP calculation has been used as input parameter), both in a non-relativistic and scalar relativistic^b run. The influence of including relativistic corrections is negligible; the energy level of the HOMO changes by roughly 0.001 Hartree (~ 0.04 eV), which is probably well below the accuracy of the computed energies. The ionization potential (corresponding to minus the orbital energy of the HOMO) is increased considerably by using the SAOP potential, which has been seen also for small molecules.

TABLE 8.1.1. ORBITAL ENERGY OF HIGHEST OCCUPIED MOLECULAR ORBITAL (A.U.)

	<i>m121h</i>	<i>m121q</i>	<i>wildtype</i>
BP/NR ^a	- ^c	- ^c	-0.0823
BP/SR ^b	- ^c	- ^c	-0.0809
SAOP/NR ^a	-0.2075	-0.2095	-0.2219
SAOP/SR ^b	- ^c	- ^c	-0.2214
GRAC/NR ^a	-0.0734	-0.0739	-0.0824
GRAC/SR ^b	- ^c	- ^c	-0.0810

a) non-relativistic results

b) scalar relativistic results using ZORA approach;

c) not performed due to experiences with *wildtype* results (see text)

As the influence of relativistic corrections is small, and the orbital energy of the HOMO in the Becke-Perdew calculation too high (too less negative), for the M121H and M121Q mutants of azurin only the SAOP potential in a non-relativistic run was used to compute the HOMO energy level, which is found to lie 0.3-0.4 eV higher than for *wildtype* eV.

Using the ionization potentials obtained with the SAOP potential as input for the GRAC potential, the fifteen lowest excitation energies for the reduced state of the proteins have been computed using TD-DFT. The values and the corresponding oscillator strengths are given in Table 8.1.2 for *wildtype*, M121H and M121Q azurin. Just as the ionization potentials of the M121H/M121Q mutants were lower than the one of *wildtype* azurin, also the lowest excitation energy is found at lower energy. Apart from the oscillator strengths, a remarkable similarity between the M121H and M121Q results is obtained.

For *wildtype* azurin the HOMO consists mainly of the p_y orbital of the Cys112 sulphur (49 %) and the d_{xy} orbital of copper (37 %). These numbers are roughly the same as the ones obtained in Section 6.3. In M121H azurin, it is no longer only the p_y orbital of sulphur that contributes (46 %), but also the p_x (3 %) and p_z (2 %) orbitals. The same goes for copper, where next to d_{xy} (26 %) also $d_{x^2-y^2}$ (7 %) contributes. This is also true for M121Q azurin, but to a lesser extent; the HOMO now consists primarily of the p_y orbital of the Cys112 sulphur (49 %) and the d_{xy} (34 %) and $d_{x^2-y^2}$ (2 %) orbitals of copper.

^a The active site of *wildtype*, M121H and M121Q consists in this chapter of the five groups coordinating to copper (Gly45, His46, Cys112, His117, Met/His/Gln121), which are cut-off at the C-alpha position

^b Scalar relativistic ZORA calculation^{117,224}

TABLE 8.1.2. EXCITATION ENERGIES (EV) AND OSCILLATOR STRENGTHS

<i>m121h</i>		<i>m121q</i>		<i>wildtype</i>	
<i>GRAC</i>		<i>GRAC</i>		<i>GRAC</i>	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
1.276	0.0055	1.252	0.0120	1.562	0.0079
1.396	0.0007	1.334	0.0002	1.691	0.0031
1.608	0.0038	1.755	0.0025	1.934	0.0169
1.652	0.0100	1.805	0.0051	2.043	0.0003
1.773	0.0024	1.844	0.0164	2.152	0.0026
1.827	0.0038	1.954	0.0016	2.238	0.0132
1.903	0.0208	1.976	0.0036	2.280	0.0007
1.927	0.0059	2.080	0.0006	2.346	0.0033
1.984	0.0132	2.113	0.0102	2.358	0.0024
2.093	0.0000	2.208	0.0008	2.455	0.0028
2.197	0.0030	2.342	0.0019	2.584	0.0080
2.260	0.0031	2.353	0.0018	2.699	0.0041
2.265	0.0041	2.511	0.0042	2.709	0.0027
2.292	0.0002	2.519	0.0015	2.776	0.0021
2.322	0.0029	2.541	0.0129	2.792	0.0013
<i>CNDO^a</i>		<i>CNDO^a</i>		<i>CNDO^a</i>	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
1.45	0.0012	1.19	0.0002	1.87	0.0005
1.65	0.0003	1.50	0.0005	1.96	0.0001
1.72	0.0001	1.66	0.0007	2.06	0.0029
1.76	0.0003	1.75	0.0002	2.08	0.0008
1.91	0.0319	1.89	0.0379	2.14	0.0041
2.02	0.0020	1.96	0.0103	2.16	0.0003
2.13	0.0050	2.12	0.0114	2.20	0.0247
2.20	0.0011	2.20	0.0294	2.30	0.0103
2.24	0.0002	2.29	0.0036	2.35	0.0025
2.31	0.0081	2.37	0.0047	2.44	0.0173
<i>INDO^b</i>		<i>INDO^b</i>		<i>INDO^b</i>	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
1.40	0.0007	1.32	0.0004	1.69	0.0004
1.63	0.0003	1.60	0.0004	1.87	0.0029
1.68	0.0002	1.67	0.0004	1.88	0.0001
1.75	0.0000	1.77	0.0001	1.92	0.0005
1.93	0.0175	2.02	0.0096	1.96	0.0001
1.96	0.0205	2.08	0.0545	2.04	0.0422
2.09	0.0070	2.21	0.0103	2.11	0.0018
2.13	0.0013	2.31	0.0059	2.13	0.0019
2.15	0.0005	2.36	0.0055	2.17	0.0050
2.24	0.0233	2.39	0.0066	2.40	0.0055

a) using "optimized" CNDO copper parameters (see text)

b) using "optimized" INDO copper parameters (see text)

In the semi-empirical (CNDO or INDO) approach, standard atomic parameters are used to calculate the one- and two electron integrals, where use has been made of empirical parameters like ionization potentials of the atoms. For organic molecules these parameters

are well calibrated and usually a good agreement between experimental and computed excitation energies are observed.

For systems containing metal atoms, a few different parameterizations are present, which were optimized for particular systems; one can be used for copper complexes with strong interactions to copper^{305,306}, while another set (Clack's values)^{307,308} was constructed for systems with weaker bonds to copper. The first set can not be used for copper proteins in the reduced state as it gives too high excitation energies: the lowest energies are found at respectively 3.07/3.16 eV (M121H), 2.66/2.79 eV (M121Q) and 3.76/3.79 eV (*wildtype*) for the CNDO/INDO Hamiltonians, which lie some 1.4-2.1 eV above the lowest DFT excitation. For the lowest ten energies, the average absolute deviation from the DFT values is 2.00 eV for CNDO and 2.08 for INDO, with a corresponding deviation for the oscillator strengths of 0.018 (CNDO) and 0.017 (INDO).

Using Clack's values^{307,308} on the contrary gives excitation energies that are lower than the DFT energies, respectively 1.12/0.76 eV (M121H), 0.93/0.63 eV (M121Q) and 1.36/1.02 eV (*wildtype*) for CNDO/INDO. However, the deviations are smaller, 0.23 (CNDO) and 0.45 (INDO) eV for the energy and 0.013/0.014 for the oscillator strength.

The copper parameters were subsequently optimized to minimize the difference with the DFT data; the final copper parameters in this adaptation process are then: $s + 9.10$ eV, $p - 42.93$ eV and $d - 15.66$ eV (CNDO) and $s - 27.57$ eV, $p - 22.16$ eV and $d + 6.27$ eV (INDO). The excitation energies when using these parameters for the reduced states of the three proteins are given also in Tables 8.1.2. In all three cases, the HOMO now consists mainly of contributions from copper (~80 %) with approximately 15 % contributions from sulphur. The average absolute deviations for the ten lowest energies are then 0.10 (CNDO) and 0.15 (INDO) eV for the energy, and 0.008 (CNDO) and 0.006 (INDO) for the oscillator strengths.

Conclusions

The excitation energies of the active site of three copper proteins in the reduced state have been computed using Density Functional Theory, using the GRAC exchange-correlation potential. The ionization potentials of the active site, which are needed for this potential, were estimated from calculations on the active site using the SAOP xc-potential, which is known to give reliable results for them.

The obtained excitation energies were used to check the performance of the semi-empirical CNDO and INDO approaches for the computation of the excitation energies, and the copper atomic parameters needed for this approaches were checked. Two sets had been previously been used on small copper complexes, which give either too high or too low excitation energies. Adapting the copper parameters improves the computed excitation energies and oscillator strengths considerably, resulting in average absolute deviations from the DFT results of 0.10/0.15 eV for the energy and 0.006/0.008 for the oscillator strength.



Oxidized states

Excitation energies of copper proteins in the oxidized state

In the previous section, the copper parameters needed in the semi-empirical CNDO/INDO methods have been calibrated for a description of the reduced state of the active sites of three copper proteins. The lowest ten excitation energies computed with these parameters showed a small difference with the corresponding reference data, which had been obtained with Density Functional Theory (DFT)¹ calculations. As was already explained in the previous section, the excitation energies of the *oxidized* state can not be obtained directly with DFT; therefore the semi-empirical (CNDO/INDO) approach is used to compute them, using a Restricted Open Shell Hartree Fock (ROHF) wavefunction followed by Configuration Interaction (CI). This method has been shown to give good results for organic molecules.³⁰⁹

TABLE 8.2.1. EXCITATION ENERGIES (EV) AND OSCILLATOR STRENGTHS USING “OPTIMIZED” (CNDO/INDO) PARAMETERS

<i>m121h</i>		<i>m121q</i>		<i>wildtype</i>	
<i>CNDO</i> ^a		<i>CNDO</i> ^a		<i>CNDO</i> ^a	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.35	0.0000	0.39	0.0000	0.31	0.0000
0.70	0.0000	0.51	0.0000	0.39	0.0000
0.97	0.0002	0.71	0.0000	0.84	0.0000
1.25	0.0001	0.88	0.0002	1.25	0.0004
3.01	0.0000	3.00	0.0000	3.07	0.0000
3.06	0.0000	3.05	0.0000	3.07	0.0000
3.10	0.0000	3.69	0.0476	3.24	0.0414
3.54	0.0403	3.86	0.0000	3.85	0.0000
3.88	0.0000	3.87	0.0000	3.90	0.0000
3.90	0.0000	4.65	0.3521	4.47	0.0002
<i>INDO</i> ^b		<i>INDO</i> ^b		<i>INDO</i> ^b	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.08	0.0000	0.09	0.0000	0.09	0.0000
0.11	0.0000	0.11	0.0000	0.09	0.0000
0.15	0.0000	0.13	0.0000	0.12	0.0000
0.20	0.0000	0.16	0.0000	0.47	0.0000
2.91	0.0000	2.89	0.0000	2.97	0.0000
2.96	0.0000	2.95	0.0000	2.97	0.0000
3.00	0.0000	3.28	0.0073	3.04	0.0063
3.09	0.0061	3.67	0.0000	3.66	0.0000
3.71	0.0000	3.69	0.0000	3.71	0.0000
3.73	0.0000	4.70	0.0000	4.84	0.3562

a) using optimized CNDO copper parameters (see Section 8.1)

b) using optimized INDO copper parameters (see Section 8.1)

The UV/Vis spectra of the oxidized states of the three proteins are characterized by intense absorptions in the visible region:²³ for M121H-azurin at 440 nm (2.818 eV) and 590 nm (2.101 eV) (with relative intensities of 2.1 vs. 1.0), for M121Q-azurin at 450 nm (2.755 eV) and 610 nm (2.033 eV) (with relative intensities of 1.0 vs. 8.0) and for *wildtype* azurin at 625 nm (1.984 eV). These absorption bands give the proteins their characteristic intense green and blue colors.

Optimized copper parameters

With the optimized copper parameters from the previous section, needed for the semi-empirical CNDO/INDO methods, the excitation energies of the oxidized state of the active site of the three copper proteins (M121H-azurin, M121Q-azurin and *wildtype* azurin) have been computed; see Table 8.2.1 for the ten lowest energies with corresponding oscillator strengths. The CNDO results with the new copper parameters (see Section 8.1) show a first excitation energy with considerable oscillation strength at too high energy (3.54, 3.69 and 3.24 eV for M121H, M121Q and *wildtype* azurin respectively) or too low wavelength (energies correspond to wavelengths of 350, 336 and 382 nm). For all three proteins, the second excitation energy with considerable oscillation strength is found at 4.64-4.68 eV (265-267 nm).

The INDO results with the new copper parameters (see Section 8.1) show a strange behavior with three to four excitation energies at very low energy (< 0.2 eV), a first excitation energy with non-zero oscillator strength at too high energy (or too low wavelength, at 401, 378 and 408 nm respectively for M121H, M121Q and *wildtype* azurin) and extremely low oscillator strength. The second excitation energy with non-zero oscillator strength is found at 4.80-4.84 eV (256-258 nm).

The excitation energies have also been computed with the CNDO/INDO methods using the standard copper parameters (see Table 8.2.2). Although there is some better agreement with the experimental data, there remains room for improvement.

Standard copper parameters

The CNDO results with the standard copper parameters result in excitation energies that are either too low (for M121H by 0.4-0.7 eV and for *wildtype* azurin by 0.1 eV) or too high (for M121Q by 0.5-0.7 eV), if the excitation energies with the largest oscillator strengths are taken into account. The relative values of the oscillator strengths corresponding to these excitations are in reasonable agreement with the experimental data²³; for M121H azurin, the two lowest excitation energies with non-zero oscillator strength, the ratio of these strengths is 1:2.5 (experimentally 1:2.1), for M121Q azurin the ratio is 3.3:1 (exp. 8:1). For *wildtype* azurin one excitation energy with a large oscillator strength is found, while a second one with almost negligible strength is observed, which agrees well with the experimental data. Finally, it should be mentioned that for M121H-azurin, a third excitation energy with significant oscillator strength is observed in the visible wavelength region (400-800 nm or 1.55-3.10 eV) at 2.45 eV with an oscillator strength equal in size to the one of the lowest excitation energy.

TABLE 8.2.2. EXCITATION ENERGIES^a (EV) AND OSCILLATOR STRENGTHS USING STANDARD COPPER PARAMETERS

<i>m121h</i>		<i>m121q</i>		<i>wildtype</i>	
<i>CNDO</i>		<i>CNDO</i>		<i>CNDO</i>	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.90	0.0007	0.78	0.0001	0.46	0.0011
1.78	0.0228	1.58	0.0027	1.86	0.1227
2.12	0.0565	2.06	0.0044	2.06	0.0128
2.45	0.0207	2.52	0.1146	2.11	0.0005
3.04	0.0000	3.01	0.0000	2.20	0.0002
3.09	0.0000	3.06	0.0000	2.87	0.0072
3.11	0.0000	3.45	0.0343	3.09	0.0000
3.40	0.0842	3.85	0.0000	3.10	0.0000
3.78	0.0003	3.87	0.0000	3.92	0.0000
3.90	0.0000	4.48	0.0083	3.97	0.0000
<i>INDO</i>		<i>INDO</i>		<i>INDO</i>	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.74	0.0016	0.71	0.0004	0.51	0.0012
1.52	0.0203	1.31	0.0017	1.67	0.1038
1.71	0.0031	1.52	0.0023	1.78	0.0391
1.86	0.0266	1.72	0.0026	1.86	0.0025
2.52	0.0773	2.48	0.1309	1.90	0.0007
2.93	0.0000	2.91	0.0000	2.78	0.0039
2.98	0.0000	2.95	0.0000	2.99	0.0000
3.01	0.0000	3.68	0.0000	3.00	0.0000
3.61	0.0020	3.70	0.0001	3.74	0.0000
3.72	0.0074	3.78	0.0270	3.79	0.0000

The INDO results with the standard copper parameters show a somewhat better agreement with experiment for M121H-azurin, with excitation energies that differ by some 0.2-0.3 eV. However, the ratio of the oscillator strengths is less well predicted at 1:2.9 (experimentally 1:2.1). The third excitation energy with significant oscillator strength that was found with the CNDO method, is not present in the INDO results. For M121Q-azurin, the two lowest excitation energies with significant oscillator strengths are found at too high energy by 0.5-1.0 eV; the ratio of the relative oscillator strength (4.8:1) is however improved in comparison with the CNDO results (3.3:1), but are still far off from the experimental data (8:1). For *wildtype* azurin, two excitation energies with significant oscillator strength are found, which are found at too low energy in comparison with experimental data, which show only one peak.

In all of these calculations, the excitation energies were calculated for the active site *in vacuo*, thereby ignoring the influence of the (protein) environment on these properties, which can have a significant effect on the energies, as has been shown for the solvent effect on the n * transition of acetone¹⁹⁶. In the next section, this influence is taken into account.

Conclusions

The excitation energies of the active site of three copper proteins in the oxidized state have been computed using semi-empirical (CNDO/INDO) methods. The influence of using the standard copper parameters or the parameters optimized for the reduced state of these proteins (see Section 8.1), has been checked. The optimized copper parameters result in excitation energies with non-zero oscillator strength that are too high in energy (CNDO), or give oscillator strengths that are too low to account for the experimentally observed bright colors.

The calculations with the standard copper parameters show better agreement with the experimental data; although the excitation energies from the CNDO method are too low or too high by 0.2-0.7 eV, the ratio of the corresponding oscillator strengths is in good agreement with experiment. The excitation energies from using the INDO method are for M121H-azurin in better agreement with experiment, but for M121Q and *wildtype* azurin the agreement is less. The ratio of the oscillator strengths is, in comparison with CNDO results, improved for M121Q azurin, but for M121H and *wildtype* azurin the agreement is less. For the latter, even two excitation energies with significant oscillator strength are observed, while experimentally only one peak is observed.

The influence of the protein environment on the energies and oscillator strengths is ignored in these calculations, but it may have a significant effect; in Section 8.3, it will be taken into account.



Protein environment

The influence of the protein and solvent environment on the excitation energies of the active site in the reduced and oxidized state

Until here in this chapter, the excitation energies were computed for the active site of copper proteins *in vacuo*, thereby ignoring the influence of the protein and solvent environment on the energies and oscillator strengths. However, it is known that the solvent effect on excitation energies can be significant, as was shown for the $n \rightarrow \pi^*$ transition of acetone in different solvents.¹⁹⁶ Similar significant effects might be expected for the presence of the protein environment surrounding the active site. Therefore the excitation energies were computed in the presence of (part of) the protein surrounding the active site, where all the residues within a radius of 10 Å from the center of the active site have been taken into account. Unlike the previous sections, where the coordinates of the active sites of the (two or four) molecules in the asymmetric unit have been averaged to obtain one active site geometry, in this section each molecule in the asymmetric unit is used separately and the energies averaged afterwards.

The calculations were performed using the Direct Reaction Field approach^{133-136,138-142}, where the active site has been treated at the semi-empirical CNDO/INDO (QM) level, the protein environment at a polarizable classical (MM) level, with a self-consistent coupling between the QM and MM systems. That is, the polarization of the QM system by the MM system, of the MM system by the QM system, and of the MM as well as the QM system by themselves are properly taken into account (see also Sections 2.3 and 2.4).

Reduced state

The computed excitation energies from the CNDO method with the optimized copper parameters for the reduced state (see Section 8.1) are given in Table 8.3.1. The presence of the protein environment brings the excitation energies down by some 0.3-0.5 eV, in comparison with the *in vacuo* results. The oscillator strengths remain more or less the same, with for M121H and M121Q-azurin the largest value found for the fifth excitation energy. For *wildtype* azurin, the excitation energy which had the largest oscillator strength (the seventh), now has almost completely lost it, and only one excitation energy with significant oscillator strength is left (the tenth).

The highest occupied molecular orbital (HOMO) consists mainly of contributions from copper (~80-90 %) with smaller contributions from the sulphur atom of cysteine 112 (~10 %) and the nitrogen atom of either one of the two histidine residues (~2 %). In comparison with the *in vacuo* results, these contributions are the same.

The INDO results show a similar behavior for the largest part of the excitation energies, but unlike the CNDO energies that were all decreased in comparison to the *in vacuo* results of Section 8.1, some INDO energies remain virtually the same, while others (for instance the lowest excitation of *wildtype* azurin) are increased in comparison with the *in vacuo* results of Section 8.1.

TABLE 8.3.1. EXCITATION ENERGIES (EV) AND OSCILLATOR STRENGTHS FOR REDUCED STATE OF ACTIVE SITES IN PRESENCE OF PROTEIN ENVIRONMENT

<i>m121h</i>		<i>m121q</i>		<i>wildtype</i>	
CNDO ^a		CNDO ^a		CNDO ^a	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
1.25	0.0010	0.90	0.0002	1.51	0.0006
1.39	0.0010	1.18	0.0001	1.62	0.0004
1.46	0.0001	1.32	0.0004	1.67	0.0018
1.50	0.0025	1.40	0.0000	1.68	0.0006
1.62	0.0175	1.62	0.0482	1.73	0.0010
1.72	0.0018	1.93	0.0052	1.83	0.0035
1.77	0.0005	2.01	0.0024	1.86	0.0006
1.81	0.0011	2.05	0.0030	1.95	0.0013
1.84	0.0043	2.16	0.0106	2.00	0.0000
1.87	0.0018	2.18	0.0077	2.10	0.0174
INDO ^b		INDO ^b		INDO ^b	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
1.40	0.0001	1.04	0.0001	1.79	0.0001
1.44	0.0002	1.09	0.0001	1.83	0.0003
1.46	0.0000	1.11	0.0000	1.86	0.0001
1.47	0.0004	1.14	0.0000	1.86	0.0002
1.52	0.0061	1.30	0.0189	1.90	0.0062
1.96	0.0006	1.90	0.0002	2.57	0.0001
1.97	0.0002	1.92	0.0001	2.63	0.0014
1.99	0.0016	1.94	0.0015	2.64	0.0005
1.99	0.0008	1.97	0.0042	2.65	0.0005
2.00	0.0044	1.99	0.0048	2.68	0.0066

a) using optimized CNDO copper parameters (see Section 8.1)

b) using optimized INDO copper parameters (see Section 8.1)

The oscillator strengths corresponding to the excitations are almost all decreased by a significant amount; for instance the fifth excitation of M121H azurin had *in vacuo* a strength of 0.018, while it is now decreased by a factor of three to 0.006. For M121Q azurin, the excitation with the largest oscillator strength is now the fifth excitation with a strength of 0.019, while *in vacuo* the largest strength was 0.054, which is also a decrease by a factor of three. For *wildtype* azurin, the factor with which the largest oscillator strength decreases is even larger (0.042 *in vacuo*, 0.007 here).

The contributions to the HOMO are, just like found with the CNDO method, mainly coming from copper itself (~90 %), with smaller contributions from Cys112 sulphur (~5-10 %) and His46/His117 nitrogens (~1 %).

Oxidized state

The excitation energies of the active sites in the oxidized state in the presence of the protein are given in Table 8.3.2. In comparison with the *in vacuo* results, a better agreement with experimental data²³ is found.

The excitations with the largest oscillator strengths from the CNDO results are observed at 2.25 and 2.88 eV for M121H-azurin, at 1.99 and 2.91 eV for M121Q-azurin and at 2.05 eV for *wildtype* azurin, which agree very well with the experimental numbers²³ of 2.10/2.82 (M121H), 2.03/2.76 (M121Q) and 1.98 (*wildtype*). Compared with the *in vacuo* results, this means a shift in energy of some 0.2-0.6 eV, either downwards or upwards. The ratio of the oscillator strengths for these excitations is in reasonable agreement with experiment, with values of 1:1.3 (M121H, exp. 1:2.1) and 4.9:1 (M121Q, exp. 8:1).

The contributions to the singly occupied HOMO are again coming mainly from copper (~70 %), with smaller contributions from Cys112 sulphur (~20-25 %) and His46/His117 nitrogen atoms (~1-5 %).

TABLE 8.3.2. EXCITATION ENERGIES^a (EV) AND OSCILLATOR STRENGTHS FOR OXIDIZED STATE OF ACTIVE SITES IN PRESENCE OF PROTEIN ENVIRONMENT

<i>m121h</i>		<i>m121q</i>		<i>wildtype</i>	
CNDO		CNDO		CNDO	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.45	0.0003	0.41	0.0000	0.51	0.0002
1.28	0.0049	1.12	0.0014	1.84	0.0004
1.46	0.0251	1.49	0.0043	2.05	0.0767
1.65	0.0122	1.91	0.0037	2.07	0.0001
2.25	0.0325	1.99	0.0757	2.23	0.0008
2.29	0.0001	2.51	0.0000	2.48	0.0003
2.34	0.0035	2.83	0.0000	2.54	0.0050
2.56	0.0000	2.91	0.0155	2.64	0.0011
2.86	0.0040	3.17	0.0000	2.88	0.0049
2.88	0.0431	3.22	0.0007	3.00	0.0006
INDO		INDO		INDO	
<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>	<i>energy</i>	<i>osc.str.</i>
0.36	0.0006	0.32	0.0002	0.38	0.0005
1.04	0.0065	0.99	0.0012	1.73	0.0030
1.13	0.0017	1.06	0.0011	1.91	0.0714
1.44	0.0197	1.30	0.0026	2.02	0.0000
2.08	0.0437	1.90	0.0896	2.12	0.0003
2.14	0.0007	2.30	0.0000	2.30	0.0077
2.38	0.0000	2.52	0.0000	2.37	0.0059
2.42	0.0003	2.86	0.0001	2.50	0.0007
2.65	0.0009	2.96	0.0014	2.62	0.0002
2.95	0.0420	3.04	0.0175	2.85	0.0002
<i>exp.</i> ²³		<i>exp.</i> ²³		<i>exp.</i> ²³	
2.10	1.0 (<i>rel.</i>)	2.03	8.0 (<i>rel.</i>)	1.98	0.05 ⁷¹
2.82	2.1 (<i>rel.</i>)	2.76	1.0 (<i>rel.</i>)		

a) using standard copper parameters

The INDO results are similar to the CNDO results, but show a slightly larger deviation from the experimental numbers²³ (see Table 8.3.2). The INDO excitations with the largest oscillator strengths are found at 2.08 and 2.95 eV (M121H-azurin), 1.90 and 3.04 eV (M121Q-

azurin) and at 1.91 eV (*wildtype* azurin), which agrees reasonably well with the experimental numbers of 2.10/2.82 (M121H), 2.03/2.76 (M121Q) and 1.98 (*wildtype*) eV. Just like was observed for the CNDO method, the presence of the protein induces a shift in the excitation energies of some 0.2-0.8 eV.

The singly occupied HOMO consists in the INDO case of contributions from mainly copper (~70 %), Cys112 sulphur (20-25 %) and His46/His117 nitrogen atoms (1-5 %).

Conclusions

The influence of the presence of the protein on the computed excitation energies and corresponding oscillator strengths is investigated using the Direct Reaction Field approach, where the active site is treated with semi-empirical (CNDO/INDO) methods and the surrounding protein and solvent environment with a polarizable classical force field.

The calculations show a negligible effect on the contributions of (copper or Cys112 sulphur) atomic orbitals to the highest occupied molecular orbital for both the reduced and oxidized state, while the excitation energies are shifted by 0.2-0.8 eV. The CNDO results are in good agreement with experimental data, both for the excitation energies as well as the corresponding oscillator strengths. The INDO results show a reasonable agreement with experiments.