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Supramolecular Catalysis

Encapsulation of a Catalytic Imidazolium Salt into Avidin: Towards the Development of a Biohybrid Catalyst Active in Ionic Liquids

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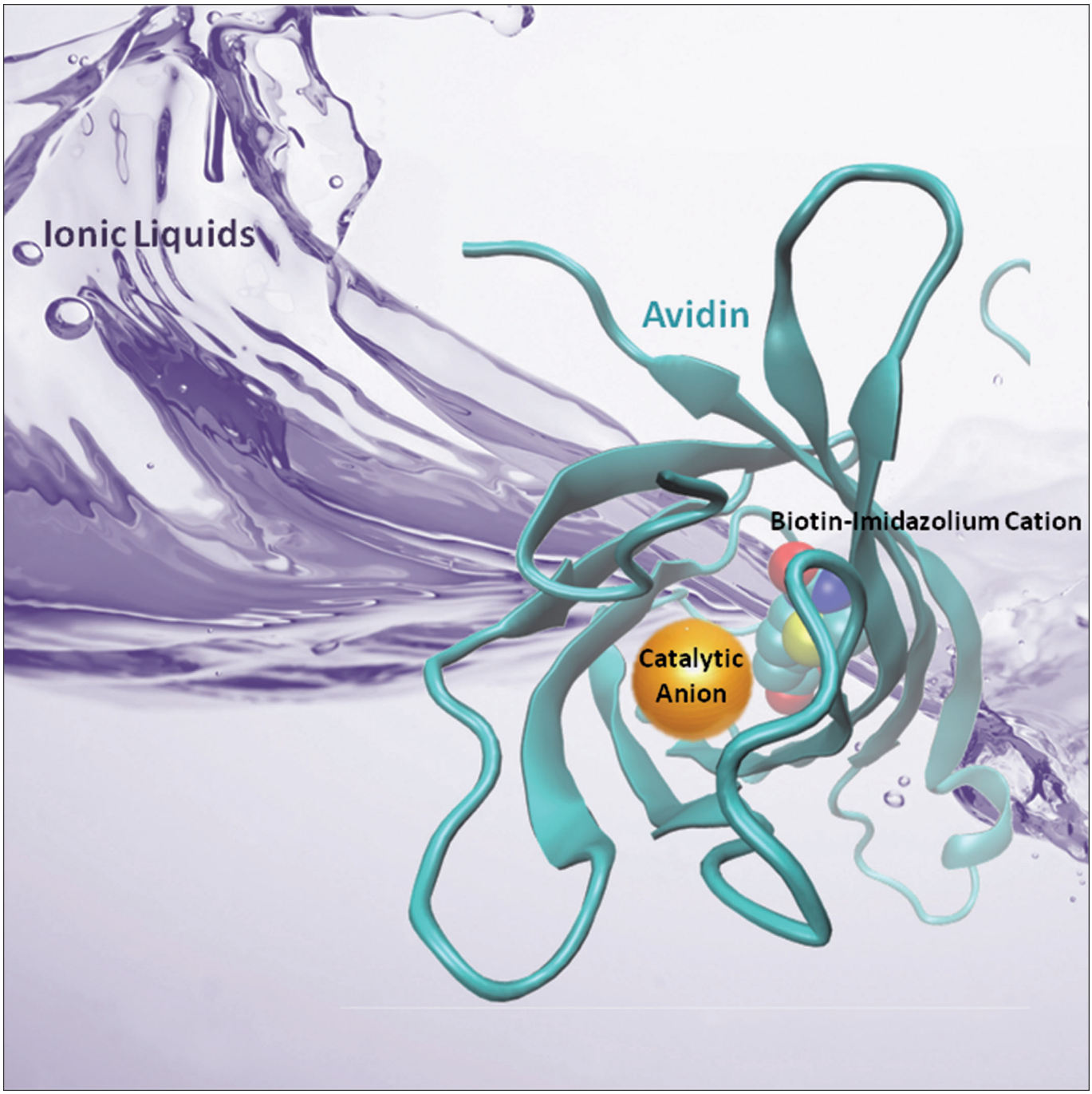

Abstract: Herein, we report the development of biohybrid catalysts that are capable of catalyzing the aldol reaction. The use of biotinylated imidazolium salts in combination with racemic or enantiomerically pure catalytic anions allowed us to study the adaptive and cooperative positioning of the anionic catalyst inside the protein. Supramolecular encapsulation of the biotinylated catalyst into avidin resulted in good selectivity for the aldol reaction performed in ionic liquid/water mixtures.

Introduction

Enzymes are evolved entities with supramolecular structures possessing highly catalytic functions, which are usually accompanied by a variety of conformational states. It has been clearly demonstrated that the motion of the enzyme structure confers catalytic efficiency during catalysis.[1] Recent progress in host–guest chemistry has allowed chemists to use noncovalent anchoring strategies to build supramolecular complexes with proteins that behave as biohybrid catalysts. These supramolecular complexes show intricate and hierarchical architectures, as well as dynamic features, all of which are required parameters for the development of catalytic systems.

Over the last decade, the development of artificial hybrid biocatalysts inspired by natural ones has been of great interest in the field of stereoselective synthesis. Ranging from synthetic macrocyclic compounds to self-assembled nanometer-sized objects, such complexes have been exploited as scaffolds to design supramolecular biohybrid systems.[2] The supramolecular anchoring strategy relies on noncovalent interactions between small molecules and the biomolecular scaffold. The crucial point of this strategy is the affinity of the guest molecule for the host biomolecular scaffold. For example, Harada et al. used the high affinity of antibodies for the creation of an artificial hydrogenase.[3] In the same spirit, Keinan et al. presented an antibody–metalloporphyrin assembly that catalyzed enantioselective oxidations.[4] In an early report, Whitesides et al. described the creation of an artificial metalloenzyme based on the very high affinity of biotin for avidin and streptavidin.[5] Since 2003, the Ward research group has intensively explored the biotin–(strept)avidin technology for the creation of artificial metalloenzymes, ranging from those mimicking natural enzymes to the design of efficient unnatural metalloenzymes.[6–10] The presence of the biomolecular scaffold offers an additional advantage for the optimization of the artificial metalloenzyme. Whereas chemical optimization can be achieved by modifying the ligand or by introducing a spacer between the biotin anchor and the metal, the biomolecular scaffold can be genetically modified, even if this requires some time through long methods in order to optimize the environment around the catalyst. Imidazolium salts, best known as ionic liquids (ILs), have gained major interest in the world of organic synthesis as promising "green solvents" as they display many interesting characteristics in terms of supramolecular architecture, nontoxicity, atom economy, and protein stabilization.[11–13] More recently, ILs have been used in asymmetric catalysis and biocatalysis, either as solvents or as actual catalysts. Their potential as solvents for the aldol reaction has been widely reported in the literature, and their superior efficiency as catalysts was recently highlighted.[14] Whereas catalysis using the cations of ILs has been widely reported in the literature, use of the anions of organic salts as catalysts is still a developing topic.[15] We previously reported the use of an imidazolium salt bearing a chiral catalytic anion that can be the source of stereoinduction in the aldol and Michael reactions.[16] Moreover, having demonstrated the beneficial effect of the second coordination sphere provided by the presence of a cyclodextrin unit in a supramolecular complex,[17] we were interested in determining the influence of a host protein on the activity and stereoselectivity of a hybrid system composed of an imidazolium-based biotinylated anchor and a nonchiral organocatalytic anion. The biotinylated imidazolium cation therefore plays an important role, not only in modulating the steric and electronic properties of the organocatalytic anion (first-coordination-sphere interactions), but also in its position inside the protein and therefore in defining the second coordination sphere.

The use of biotinylated imidazolium salts in combination with racemic or enantiomerically pure anions allowed us to study the adaptive and cooperative positioning of the anionic catalyst inside the protein (Scheme 1). We report the preparation of a new type of biohybrid catalysts active in IL/H_2O mixtures and mechanistic insights into its organocatalysis by using the aldol reaction as an example. To the best to our knowledge, this is the first example of a biohybrid catalyst that is able to function in an ionic liquid medium.

Results and Discussion

Avidin is a glycosylated protein that is naturally present in egg white.[18] Avidin is a tetrameric eight stranded β-barrel protein that binds up to four biotins with high affinity. The interaction between biotin and avidin is extremely tight with $K_a = 1.7 \times 10^{15}\ M^{-1}$. This high value ensures a quasi-irreversible anchoring of biotinylated compounds in the protein pocket as a result of numerous interactions between the biotin and the protein, such as hydrophobic interactions,[19] Van der Waals interactions,

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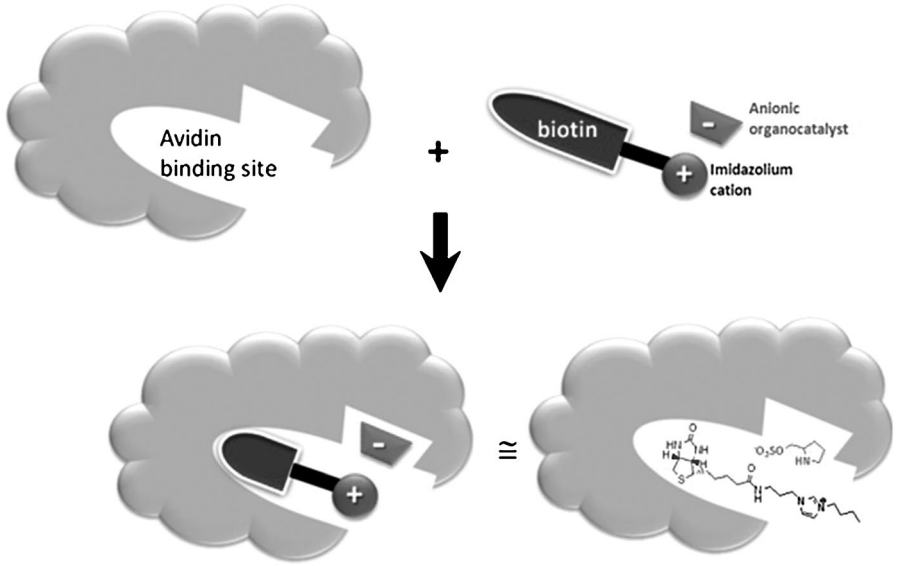


Scheme 1. Supramolecular encapsulation of the biotinylated catalyst in avidin.

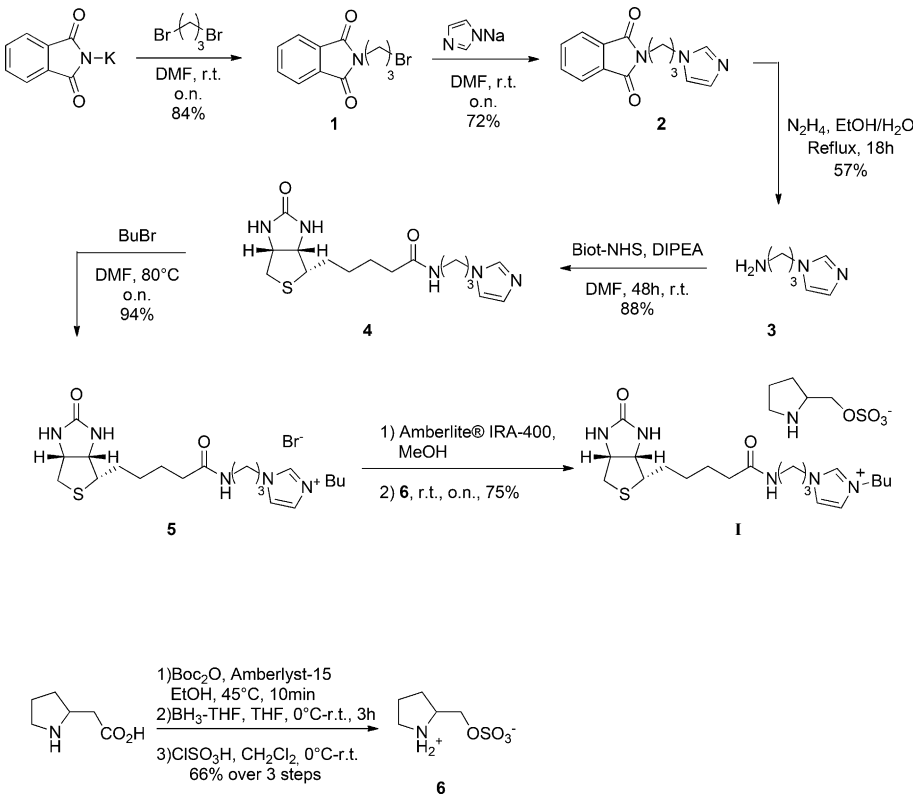
and hydrogen bonding.[20] Avidin is extremely robust and stable at high temperatures,[21] at extreme pH,[22] and at high concentrations of denaturating agents.[23] The robustness of this protein allows its use as a scaffold for a wide range of applications, which also rely on the fact that the valeric acid side chain of biotin can be derivatized with little effect on the remarkable affinity of the biotin–avidin complex.[24]

The synthesis of the cationic anchor began with the formation of the imidazole-amine **3** through a typical Gabriel synthesis. The coupling of **3** with the activated (+)-biotin afforded precursor **4**, which was then alkylated with 1-bromobutane under standard conditions to obtain the air-stable biotinylated imidazolium salt **5** (Scheme 2). As we have previously reported, anion metathesis of zwitterion **6** and bromide **5** required the formation of a non-isolable hydroxide intermediate by using an IRA-400 ion exchange resin.[16] The biotinylated imidazolium salt **I** containing the pyrrolidine anion was obtained by simple anion exchange between the hydroxide anion and zwitterion **6**. A control experiment with **I** as the catalyst was performed to assess its activity in the absence of avidin. The desired aldol products were obtained with more than 98% conversion under typical aldol conditions by using cyclohexanone as the solvent.[16]

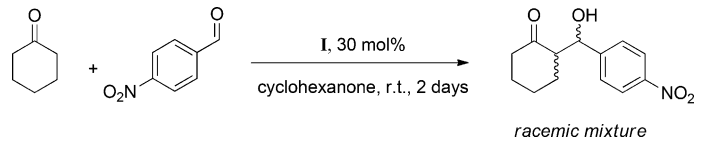
The formation of a racemate in this case also demonstrated the lack of assistance of **I** in the stereoselective control of the aldol reaction (Scheme 3). To obtain homogeneous conditions for the aldol reaction, with avidin being extremely hydrosoluble, the reaction medium was partitioned between water and an organic co-solvent, where all the reactants were soluble.

No denaturation of avidin was expected under these reaction conditions, as the stability of its complex with (+)-biotin was previously described.[25] The formation of the biohybrid catalyst **I·Av** was performed in situ by mixing **I** and avidin for 30 min at room temperature. The chiral induction brought about by the avidin was first studied under un-optimized catalytic conditions (Scheme 4). Biohybrid catalyst **I·Av** was active in the aldol reaction performed in H_2O/MeOH 4:1 mixture, but only a small enantiomeric excess (*ee*) was observed for the formation of the major *anti* diastereoisomer (Scheme 4).

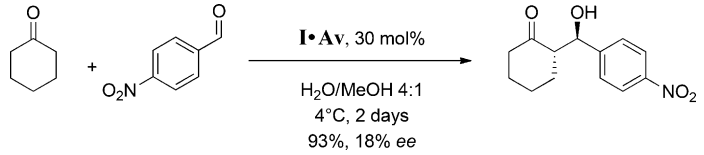
For all the catalytic tests, avidin was used in a small excess (1.15 equiv for 1 equiv of **I**) to ensure the complete binding of



Scheme 2. Synthesis of the biotinylated catalyst. Overnight = 16 h.



Scheme 3. Initial reaction conditions for the aldol reaction.



Scheme 4. Reaction conditions using **I·Av** as the catalyst.

I. Based on these preliminary results, we evaluated the solvent effect on the reaction by screening several organic solvents and ILs (Table 1). When using water-miscible solvents such as MeOH, DMF, and DMSO (entries 1–3), excellent conversions were obtained after 48 h of reaction.

Hydrophobic solvents like AcOEt or CH_2Cl_2 (entries 4–5), predictably, led to lower conversion rates, the reactants not being soluble in water. Carrying out the reaction in H_2O/cyclohexanone led to complete conversion after two days. Despite an excellent conversion rate, the enantioselectivity was still low (under 20%). These results were improved only when we used ionic liquids as co-solvents. Different 1-butyl-3-methylimidazolium salts ([Bmim]X) were tested, and whereas the conversion rates were lower than those obtained in common organic solvents, the selectivity was improved to up to 28% *ee* when using the water-soluble [Bmim]Br. Replacement of [Bmim]Br with the highly hydrophobic [Bmim]NTf_2 resulted in a decrease of enantioselectivity (down to 4%, entry 9). The decrease in conversion when using ILs could be associated with the high viscosity of the IL at low temperatures. Moreover, the nature of the solvent did not seem to affect the diastereomeric ratio (d.r.). In an attempt to understand the effect of ILs on the selectivity of the aldol reaction, the ratio of [Bmim]Br in the reaction medium was incrementally varied. As shown in Figure 1, a linear increase in the selectivity for the major *anti* diastereomer was obtained by increasing the amount of IL up to 80% v/v, where 70% *ee* was obtained. Such linearity in the variation of enantioselectivity suggests a possible rearrangement of the avidin structure with increasing IL content. When going beyond a 4:1 IL/water ratio, the enantioselectivity decreased to 24% *ee* and no reaction occurred when the reaction was performed in pure [Bmim]Br, proving that a small amount of water was necessary to completely solubilize the avidin and maybe to preserve its 3D structure. To the best of our knowledge, there are no reports in the literature on the conformational changes of avidin in an ionic liquid medium. We performed circular dichroism (CD) measurements of avidin (Figure 2, solid line) and **I·Av** (dotted line) in water and a 4:1 IL/water mixture. As shown in Figure 2, substantial changes in avidin's conformation can be observed in the IL/water mixture. A significant difference between the spectra of both free avidin and **I·Av** revealed a conformation change in both secondary (far-UV spectrum) and tertiary (near-UV spectrum) structures of avidin. The distinctive peaks for β-sheets at 197 nm (positive band) and 213 nm (negative band) in water were replaced by a negative absorption band of high magnitude at 203 nm in the IL/water mixture, suggesting a possible appearance of random coil structures. The broad peak around 260 nm in the near-UV IL/water spectrum suggests that the intra-monomeric disulfide bond between Cys-4 and Cys-83 was reduced.[26] Further studies must be performed to elucidate if specific structure changes occur and to see if a new inter-monomeric disulfide bond is formed under these conditions.

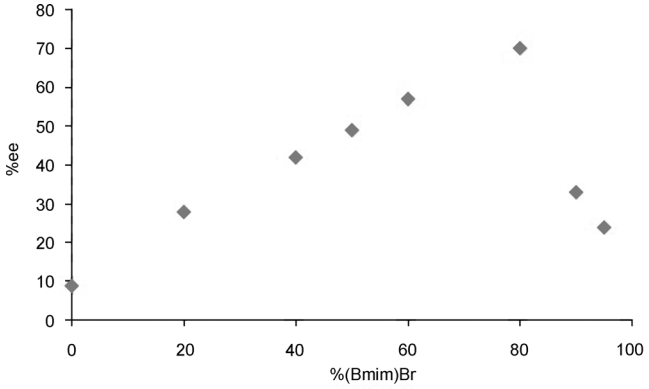
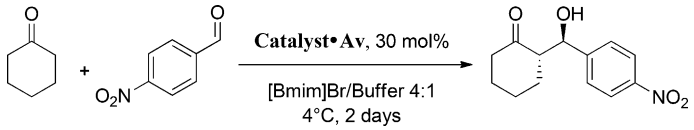


Figure 1. Influence of the ionic-liquid content on the enantioselectivity of the reaction.

These conformational changes in avidin structure may be responsible for the variation in the selectivity of the reaction arising from the differing IL content. As previously reported, the binding of **I** has no significant effect on the structure of avidin in water.[27–28]

Table 1. Solvent screening.



Entry	Solvent (4:1)	Conversion [%][a]	d.r. (*syn*/*anti*)[a,b]	*ee* (*syn* [%]/*anti* [%])[b]
1	H_2O/MeOH	93	33:66	rac.:18
2	H_2O/DMF	94	41:59	rac.:12
3	H_2O/ DMSO	95	41:59	rac.:11
4	H_2O/AcOEt	31	34:66	4:16
5	H_2O/CH_2Cl_2	21	49:61	10:14
6	H_2O/cyclohexanone	>99	39:61	rac.:20
7	H_2O/[Bmim]Br	47	41:59	6:28
8	H_2O/[Bmim]BF_4	43	37:63	14:26
9	H_2O/[Bmim]NTf_2	46	41:59	18:4

[a] d.r. = diastereomeric ratio. [b] Determined by chiral HPLC.

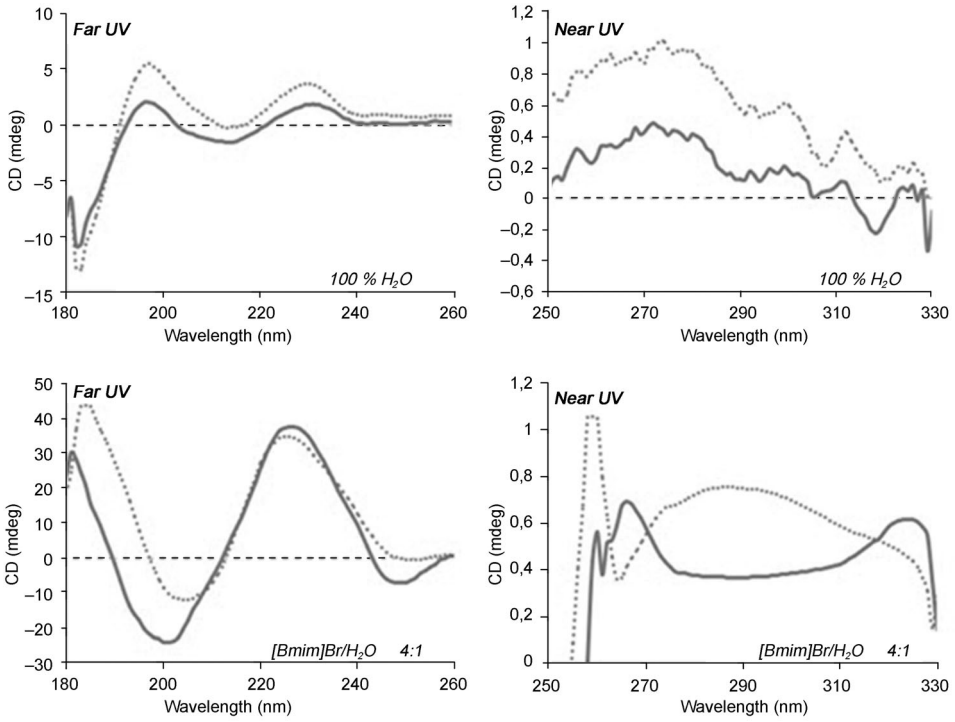


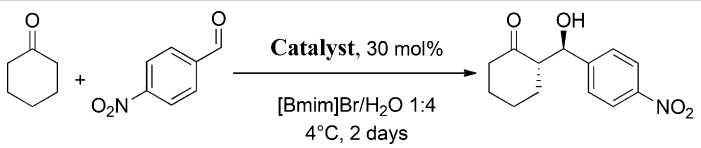
Figure 2. Circular dichroism spectra of avidin (solid line) and **I·Av** (dotted line) at 0.5 mg mL^{-1}.

However, in the IL/water mixture, an important structural change of avidin was observed in both the far- and near-UV spectra when **I** was encapsulated in the protein.

At this point, several control experiments were carried out to assess the role of each component of the reaction (Table 2). A first reaction was performed under the optimized conditions with **5·Av** instead of **I·Av**, but no traces of the aldol products were observed, highlighting the importance of the counter anion in the catalytic process (Table 2, entry 1). One important observation is that avidin, exhibiting a molecular weight of approximately 88 kDa, working in high substrate dilution conditions was necessary. Since avidin exhibits a molecular weight of approximately 88 kDa, substrate in high dilution was necessary to ensure full solubilization of the protein and to lower the viscosity of the reaction media; these high-dilution conditions were approximately 100 times lower than those usually described in the literature. A control experiment was carried out under these dilution conditions with **I** as the catalyst in the absence of avidin. No traces of the aldol products were observed after 48 h, confirming that the catalytic activity of **I** outside avidin was negligible in these high dilution conditions (Table 2, entry 2). This experiment also allowed us to discount any possible interference by competitive reactions catalyzed by the free catalyst **I** outside the avidin active site. These results were also proof of the supramolecular complexation between **I** and avidin, showing that encapsulation of **I** inside avidin is required for the reaction to occur in an acceptable time frame. A catalytic run was also performed with zwitterion **6** with avidin to test the possible complexation of the anionic catalyst with any charged residue inside avidin (Table 2, entry 3). No conversion was observed, implying that the absence of the imidazolium moiety prevents the correct encapsulation of the catalytic anion inside the avidin pocket. A final control experiment was performed to determine whether avidin in the 4:1 IL/water mixture exhibits any catalytic activity (Table 2, entry 4). Once more, no conversion was observed after 48 h.

As pH has a major impact on the behavior of proteins in aqueous media, several tests were performed using different buffers in the IL mixtures, allowing the pH to be varied from 1 to 12. Carrying out the reaction at pH 1 led to the degradation of **I·Av**, preventing catalysis (Table 3, entry 1). Unsurprisingly, pH has a dramatic influence on the catalyst efficiency: at higher pH, the conversion was higher, but the selectivity was lower. By plotting the diastereomeric ratio versus pH, it can be seen that the overall trend shows that a more basic medium leads to a decrease of enantioselectivity, but also gives rise to the proportion of the *syn* isomers (Figure 3). The hypothesis that a possible base-catalyzed reaction was being carried out outside avidin was confirmed when a blank experiment without avidin was performed using NaOH as the sole catalyst (Table 3, entry 13). A racemic mixture of aldol products was obtained, indicating that the drop in selectivity observed at high pH results from competition between the reaction occurring inside the protein and the base-catalyzed reaction happening outside avidin, where there is no stereocontrol. As low pH is required to favor the "inside" reaction, we chose to keep the pH of the reaction medium at 3. The problem of low conversion at this pH was solved by increasing

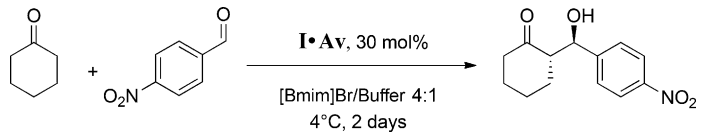
Table 2. Control experiments.



Entry	Catalyst	Conversion [%]
1	**5·Av**	–[a]
2	**I**, no avidin	–[a]
3	**6**, with avidin	–[a]
4	avidin only	–[a]

[a] No conversion was observed by HPLC.

Table 3. Influence of pH.



Entry	pH[a]	Conversion [%][b]	*ee* (*anti*) [%][b]
1	1	–	–
2	2	43	61
3	3	43	70
4	4	38	63
5	5	41	59
6	6	69	42
7	7	81	27
8	8	89	22
9	9	86	17
10	10	89	13
11	11	69	16
12	12	85	10
13[c]	NaOH 0.1 M	>99	rac.

[a] pH 1–2 and 12, chloride buffer; pH 3–6, acetate buffer; pH 7–8, phosphate buffer; pH 9, trishydroxymethylaminomethane (TRIS) buffer; pH 10–11, carbonate buffer. [b] Determined by chiral HPLC. [c] No avidin was added.

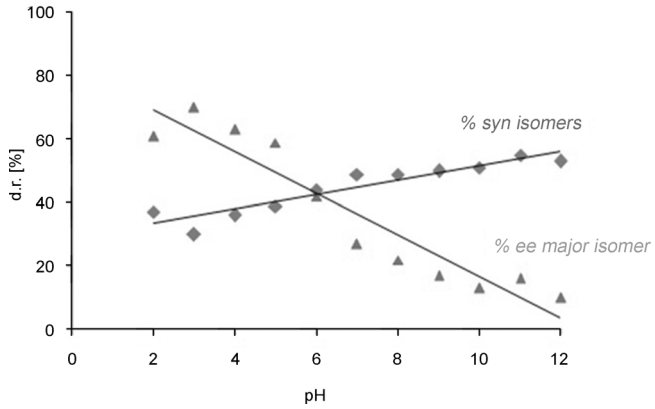


Figure 3. Trends in selectivity with varying pH.

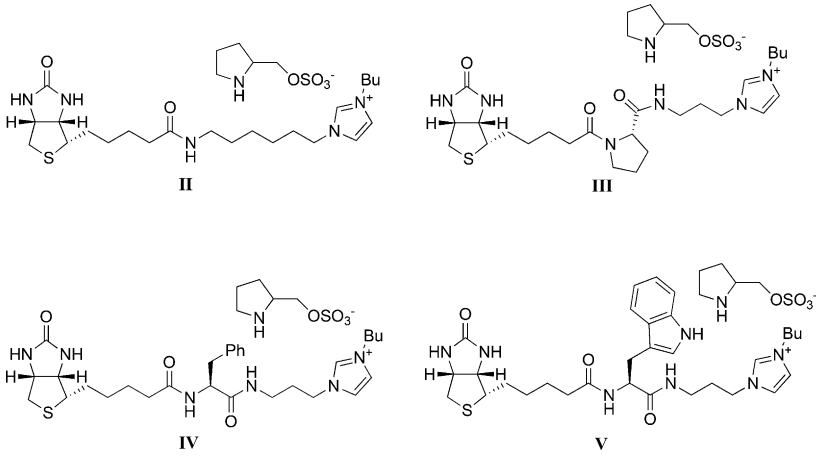
how these locations could influence the reactivity of the catalyst.

Ward et al. recently reported that the insertion of an amino acid in biotinylated catalysts increased the performance of their metalloenzymes.[29] Based on these observations, we speculated that: 1) Aromatic amino acids with hydrophobic side chains (L-Phe and L-Trp) would display π–π interactions with hydrophobic residues inside the cavity; 2) Adding a longer alkyl chain between the biotin moiety and the imidazolium cation would allow more flexibility inside the protein; 3) L-Proline, with its high conformational rigidity, would restrain the liberty of movement of the anionic catalyst. All *tert*-butoxycarbonyl (Boc)-protected amino acids were first coupled with **3** through a simple and efficient procedure using *N*-hydroxysuccinimide/dicyclohexylcarbodiimide (NHS/DCC) in acetonitrile. A simple deprotection step, followed by the coupling of (+)-biotin *N*-hydroxysuccinimide ester, led to the imidazole precursors. Alkylation using 1-bromobutane, followed by anion metathesis with **6** afforded **II**, **III**, **IV**, and **V** as air-stable compounds. Full experimental details for the synthesis of these compounds can be found in the Supporting Information. The formation of the biohybrid catalysts was performed in situ and catalysis was carried out by using the optimized conditions already described. The catalytic activity of **II–V** and their contributions to the stereocontrol of the reaction were also assessed with avidin-free catalytic runs. All results are shown in Table 4.

As previously observed, no chiral induction and extremely low conversions were obtained for all the tested catalysts in the absence of avidin, owing to the extremely diluted conditions. The lack of reactivity underlines the crucial role of avidin in terms of acceleration of the reaction rate. The variation of the structure of the cationic thread did not affect the complexation efficiency between the catalyst and avidin. However, no significant variation in the obtained *ee* was observed when using different cationic threads. These results suggest that the pyrrolidine moiety has enough freedom of motion inside the

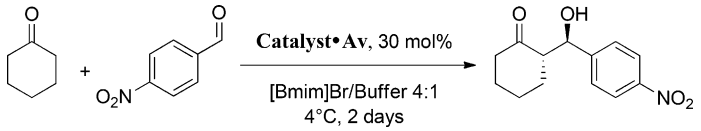
the substrate concentration to 5×10^{-2} M, leading to 94% conversion after 48 h with the same enantioselectivity.

To gain a better understanding of the interactions between the biotinylated imidazolium salt and avidin, as well as the mobility of the catalytic anion in avidin's binding site, we designed and synthesized different cationic threads (Scheme 5). The structure of the biotinylated thread was modified in an attempt to position the anionic catalyst at different locations inside the avidin binding site and to see



Scheme 5. Variation of the structure of the cationic scaffold.

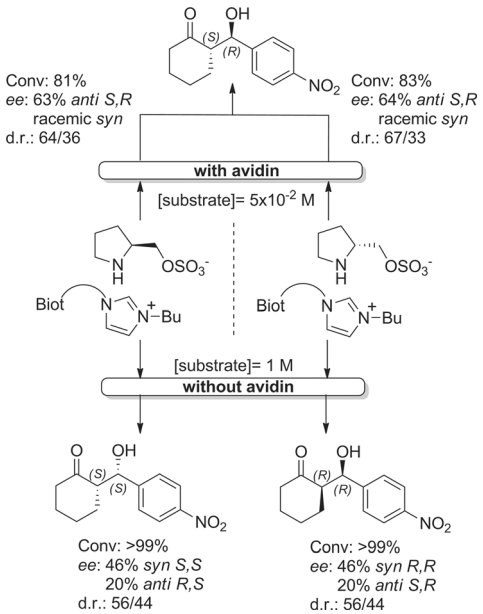
Table 4. Effect of the cationic scaffold.[a]



Entry	Catalyst	Conversion [%][b]	*ee* (*anti*) [%][b]
1	**I·Av**	94	70
	I	<1	<3
2	**II·Av**	88	68
	II	<2	<4
3	**III·Av**	89	66
	III	<1	<4
4	**IV·Av**	86	66
	IV	<1	<4
5	**V·Av**	88	66
	V	<2	<5

[a] Substrate concentration: 5×10^{-2} M in each case. [b] Determined by chiral HPLC.

avidin cavity to adopt the same optimized position in all these cases, independent of the structure of its cationic counterpart. These results also show that the cation does not actively participate in the catalytic process and that the movement of the anion inside the avidin pocket is the crucial parameter of this biohybrid species. To support these observations, the two enantiomers of the 2-pyrrolidinemethanesulfonate salt of **6** were prepared. Each catalyst bearing a chiral counter anion was tested under the optimized conditions and this, surprisingly, led to similar results compared with their racemic counterparts in the presence of avidin (Scheme 6). Interestingly, the D-proline derivative did not give the expected enantiomer of the usual *anti* aldol product, but gave the (*S*,*R*) enantiomer with an enantiomeric excess similar to the results obtained by using **I**.



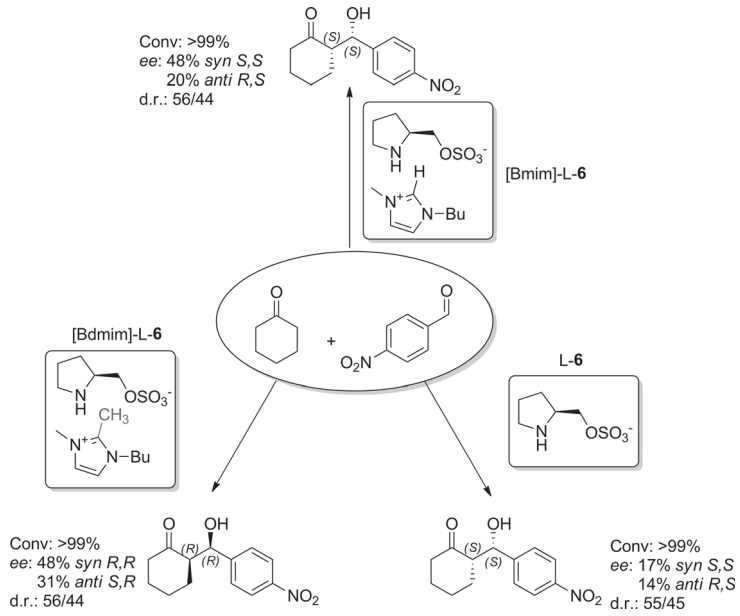
Scheme 6. Influence of the chirality of the anion.

To get a better insight into the impact of the intrinsic chirality of the anion, the chiral diastereomeric analogues of **I** bearing L- and D- pyrrolidinemethanesulfonate anions were tested in the absence of avidin, using an appropriate substrate concentration (1 M). The results are compiled in Scheme 6, where only the major diastereomers of the obtained aldol products are shown. This time, the *syn* diastereomers were the major product and the selectivities depended on the absolute configuration of the anion, each anion favoring one enantiomer of the *anti* products. However, the enantiomeric excess for the *anti* diastereomer was very low (20% *ee* max.). Two conclusions can be drawn from these results: 1) There is no obvious match–mismatch evidence in the case where the racemic anion of **I** is embedded in the protein, as both versions of the catalyst led to similar results in the formation of the (*S*,*R*) *anti* enantiomer (Scheme 6); 2) Even if the intrinsic chirality of the anion is directly responsible for the stereoselective outcome of the reaction in the absence of avidin, it seems that the chirality inside the protein governs and improves the selectivity of the biohybrid catalyst.

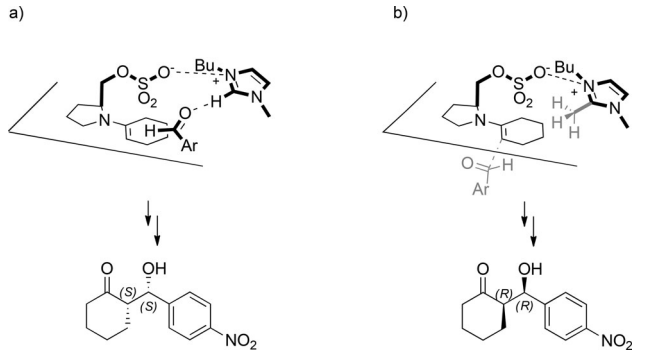
As both enantiomers of the *syn* aldol product can be obtained in similar excess regardless of whether the L- or D-pyrrolidinemethanesulfonate anion is used, it is clear that the sulfonate moiety dictates the approach of the aldehyde towards the enamine. Complementary experiments were carried out with three non-biotinylated catalysts to understand the role of both the anion and cation in the model aldol reaction (Scheme 7). The presence of the imidazolium cation close to the sulfonate group favors the approach of the aldehyde through hydrogen bonding of H-2 of the imidazolium to the oxygen atom of the aldehyde. Steric hindrance results in attack of the enamine on its *Si* face, leading to the formation of the *syn* diastereomer. When the imidazolium H-2 is replaced by a methyl group, the possibility of hydrogen bonding is removed and the selectivity of the reaction is ruled purely by steric restrictions; thus, the aldehyde is forced to approach the "opposite face" of the enamine (Scheme 8).

The importance of the imidazolium cation was also highlighted by the results obtained when using the deprotonated version of **6** as the catalyst; in this instance, the selectivity decreased considerably.

While it is still unclear why the *anti* diastereomer was preferentially formed when the reaction was carried out inside avidin, one could suppose that the sulfonate group can interact with a cationic residue inside avidin, resulting in a conformational change to the sulfonated arm. In this case, the steric hindrance initially caused by the catalyst would play less of a role in the discrimination of the enamine faces and the observed stereocontrol would be induced by the chiral pocket of the avidin (for more details see the Supporting Information). This hypothesis is also consistent with the results obtained with the different enantiomerically pure anions, which gave the same aldol product. These results also suggest the possibility of free motion of the anions inside avidin and show that racemic anions can be used to form the diastereomeric cata-



Scheme 7. The role of the imidazolium cation in the catalytic process.



Scheme 8. Possible transition states for the aldol reaction performed with a) [Bmim]-L-**6** and b) [Bdmim]-L-**6**.

lyst. Combined with its ionic liquid compatibility, this could be an asset for the preparation of substrate-tolerant biocatalysts without additional optimization of the avidin active site. Moreover, current work in our group aims at the synthesis of new chiral pyrrolidine-based anions to achieve a fine tuning and a better understanding of this system in catalysis, especially for the development of new reactions.

Conclusion

We presented the assembly of a biohybrid catalyst obtained by the complexation of biotinylated imidazolium salts with racemic or enantiomerically pure catalytic anions and their activity in the aldol reaction performed in IL/water mixtures. Circular dichroism studies revealed the influence of the ionic liquid on the conformation of avidin and the biohybrid catalyst. The high degree of freedom of the anionic catalyst inside the protein was demonstrated for different biohydrid catalysts and the influence of the second-sphere coordination brought about by the protein around the catalyst was discussed. Further studies on the substrate tolerance of our system are currently under investigation in our group and efforts are currently being made towards the synthesis of new catalytic anions with different structures to improve the catalyst's activity. Also, the design of novel anionic compounds that can act as ligands for transition metals could lead to the assembly of new biohybrid catalytic systems.

Experimental Section

All organic compounds were purchased in their highest available purity and used without further purification. Egg-white Avidin was purchased from Lee Biosolutions. NMR experiments were recorded on an Avance 300 Brucker, at 300 and 75.5 MHz, and an Avance 400 Brucker, at 400 and 100 MHz, with non-spinning samples. All NMR experiments were obtained by the use of the commercially available sequence on Brucker spectrometers. Coupling constants are given in Hertz (Hz) and chemical shifts are given in ppm (δ) and measured relative to residual solvent. Mass spectral data were obtained by the Université de Montréal Mass Spectrometry Facility and were recorded on a Mass spectrometer TSQ Quantum Ultra (Thermo Scientific) with accurate mass options instrument.

Typical procedure for the Aldol reaction

Stock solutions of starting materials and catalysts were prepared: $\mathbf{S_1}$ refers to a 0.2 M solution of *p*-nitrobenzaldehyde in [Bmim]Br; $\mathbf{S_2}$ refers to a 2 M solution of cyclohexanone in [Bmim]Br; $\mathbf{S_3}$ refers to a 0.03 M solution of catalyst in water/buffer. In a vial, avidin (12 mg, 13.8 U/mg, binds 0.678 μmol of biotinylated catalyst, 1.13 equiv compared with catalyst) was suspended in [Bmim]Br (60 μL). $\mathbf{S_2}$ (10 μL, 20 μmol, 10 equiv) and $\mathbf{S_3}$ (20 μL, 0.6 μmol, 30 mol%) were added. Once the avidin was completely dissolved, the reaction mixture was stirred for 30 min at 23 °C to allow the formation of the catalytic species. $\mathbf{S_1}$ (10 μL, 2 μmol, 1 equiv) was added and the reaction mixture was stirred for 48 h at 4 °C. Diethyl ether (0.5 mL) was then added and the vial was vortexed for 1 min. The ether phase was analyzed by chiral HPLC (ChiralPak AD-H column, hexanes/isopropyl alcohol (IPA) 95/5, 0.5 mL min^{-1}, $\lambda = 254$ nm) t_R (*anti* isomer) = 40.84 (minor), 56.69 (major), t_R (*syn* isomer) = 27.96 (minor), 37.35 min (major).

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