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COMMUNICATION

Aldolase activity of serum albumins†

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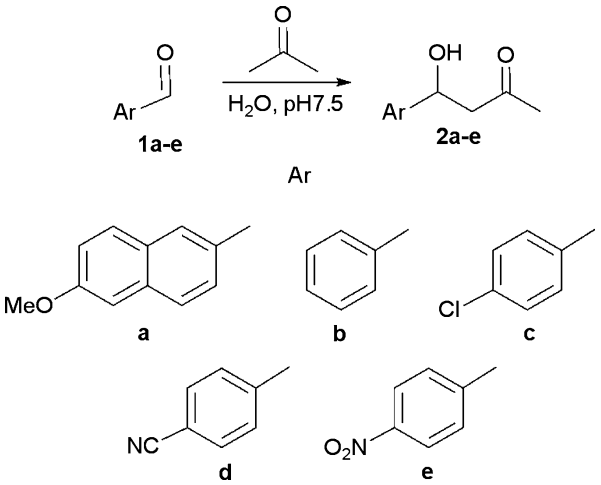
Bovine and human serum albumins catalyze the aldol reaction of aromatic aldehyedes and acetone, with saturation kinetics and moderate and opposite enantioselectivity. The reaction occurs at the binding site in domain IIa, and is inhibited by warfarin. Kinetic data are consistent with an enamine mechanism. The activity is conserved in a 103 aminoacid peptide derived from the albumin sequence.

The aldol reaction is one of the most important methods of organic synthesis, allowing formation of a carbon–carbon bond with generation of one or two stereocenters.[1] Stereocontrol can be achieved by asymmetric induction,[1] by chiral auxiliaries,[2] and by asymmetric catalysis.[3] The latter approach is very attractive and great effort has been dedicated to the discovery of new catalysts, including small organic molecules (organocatalysts),[3,4] chiral metal complexes,[2,3] enzyme mimics as catalytic antibodies[1,5] and polymers.[6]

Covalent catalysis is used by class I aldolases,[5] and also by designed enzymes[7] and catalytic antibodies.[8] A common feature found in the catalytic site of these proteins is the presence of a low pK_a, nucleophilic lysine, surrounded by a hydrophobic environment, which catalyzes the aldol/retroaldol reaction by an enamine/Schiff base mechanism.[5,7,8]

A low pK_a lysine is also present in the IIa binding site of human (HSA: Lys-199) and bovine (BSA: Lys-222) albumin. This residue is involved in the covalent binding of substrates,[9] and is responsible for albumin's ability to behave as an enzyme-like catalyst in β-eliminations,[10] in the decomposition of Meisenheimer adducts,[11] and in the Kemp elimination.[12] Notwithstanding the presence of an active lysine in a hydrophobic binding site, which might present albumin with a chance to behave as an aldolase catalyst, BSA was reported by Barbas not to catalyze the aldol addition of acetone to 3-(4-acetamidophenyl)-propanal.[13] However, based on albumin's known specificity for aromatic molecules,[9a,14] we reasoned that the lack of catalytic activity observed by Barbas might be due to poor recognition of the aliphatic aldehyde by the protein and decided to further investigate the reaction. We now report that the aldol addition of acetone to aromatic aldehydes (Scheme 1) is indeed accelerated by albumin with an enzyme-like mechanism.



Scheme 1 Aldol reactions of aldehydes **1** with acetone.

Formation of aldol **2a** by reaction of acetone with aldehyde **1a** is accelerated by both HSA and BSA, with saturation kinetics (Fig. 1; Table 1). The kinetic parameters are very similar for the two proteins, the catalyzed process being three orders of magnitude faster than the uncatalyzed one, while the Michaelis–Menten constant is in the millimolar range in both cases. The reactions exhibited multiple turnover, thus revealing true catalysis, and allowing full conversion of **1a** to aldol **2a** (partial dehydration of **2a** to the α,β-unsaturated ketone was observed at over 80% conversion).

Albumin-catalyzed reactions show an opposite enantiopreference: in the BSA-catalyzed process, the Si face of aldehyde **2a** is preferred, thus giving the *S* aldol with 40% ee. On the contrary, the HSA-catalyzed reaction leads to the *R* product with 60% ee. The stereoselectivity is moderate if compared to the 97% displayed by antibody 38C2 in the same reaction,‡ but significantly higher than the 8% ee obtained with Tanaka's catalytic peptides in the reaction of acetone and **1e**.[16] The switch in enantioselectivity going from BSA to HSA might reflect the different position of the lysine residue (222 in BSA and 199 in HSA) within the otherwise identical sequence of the IIa binding site, thus suggesting an active role for this aminoacid in the catalytic mechanism.

BSA showed no aldolase activity after reaction with fluorescein isothiocyanate. This reagent selectively modifies Lys-222,[11c] thus

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† Electronic supplementary information (ESI) available: HPLC and kinetic data, stereoselectivity, Hammett Plot, sequence of IIa103 peptide and details of its preparation and purification, DFT geometries of transition states. See DOI: 10.1039/c0ob01219j

Table 1 Kinetic parameters (at 37 °C) for the uncatalyzed and albumin-catalyzed aldol reactions

Entry	Substrate/albumin	k_{unc}[a] 10^3 [min^{-1}]	k_{cat}[b] 10^3 [min^{-1}]	k_{cat}/k_{unc}	K_M[b] [mM]	$k_{cat}/K_M k_{unc}$ [M^{-1}]	ee[c] %
1	**1a**/HSA	0.00804	10.0	1250	2.1	4.1×10^5	60 (*R*)
2	**1a**/BSA		10.4	1290	3.3	2.4×10^5	40 (*S*)
3	**1b**/BSA	0.0326	6.72	206	4.3	4.8×10^4	
4	**1c**/BSA	0.258	23.6	92	2.7	3.4×10^4	
6	**1d**/BSA	1.62	427.1	263	22.3	1.2×10^4	
7	**1e**/BSA	2.39	672.0	280	66.1	4.2×10^3	

[a] observed pseudo first order values. [b] apparent values in 10% aqueous acetone. [c] at 70% conversion by $Eu(hfc)_3$ NMR; abs. configuration assigned by comparison with that of **2a** obtained by antibody 38c2 catalysis.[15]

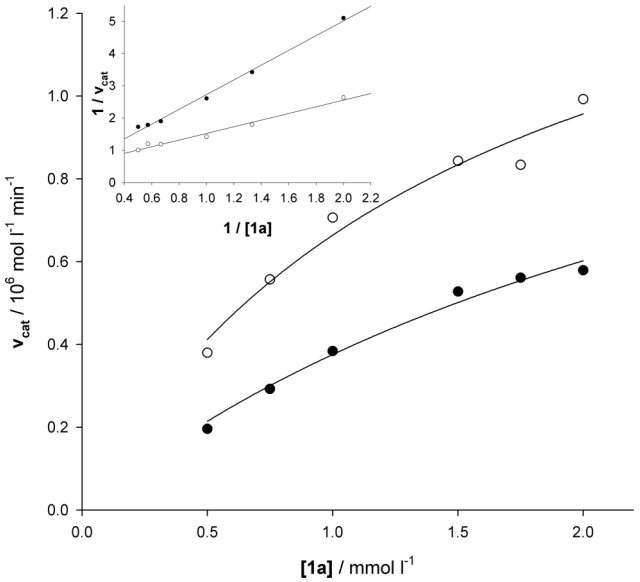


Fig. 1 Michaelis–Menten and Lineweaver–Burk (insert) plots for the BSA (●) and HSA (○) catalyzed aldol reactions of aldehyde **1a** and acetone.

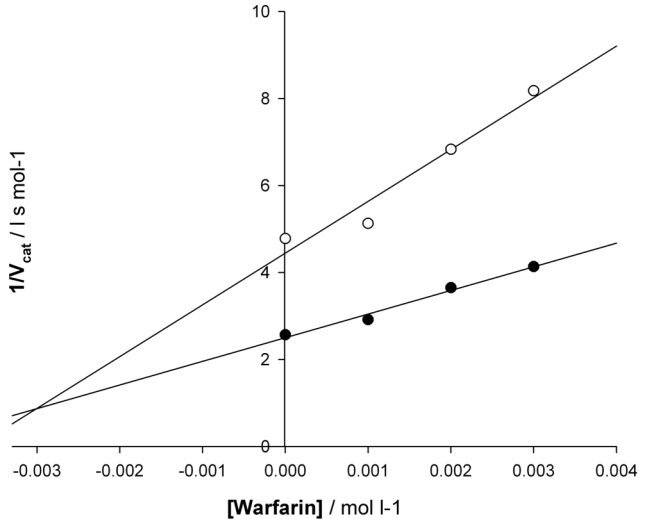


Fig. 2 Dixon plot for the warfarin-inhibited aldol reaction of aldehyde **1a** and acetone. The concentrations of **1a** are 0.5 mM (●) and 1 mM (○).

strongly supporting the hypothesis that this residue plays a key role in the catalytic mechanism. In order to further confirm this, the reaction was studied in the presence of (±)-warfarin, a well-known ligand of the IIa site.[17] BSA's aldolase activity is competitively inhibited by the drug, with a Ki of 2.94 mM (Fig. 2). This experiment not only confirms that the small molecule binding site of subdomain IIa is the aldolase site, but also provides clear evidence in favour of a specific catalytic process.

BSA proved active on the whole set of *p*-substituted benzaldehydes **1b–e** (Table 1), although worked less efficiently than with aldehyde **1a**, with k_{cat}/k_{unc} ratios in the 100–300 range. Similar values of K_M were found for aldehydes **1a–c**, while K_M increases by one order of magnitude when the aldehyde bears a polar group in the *para* position (**1d**, **1e**). This is consistent with the topology of the IIa binding site,[9a] which is sock-shaped, with the bottom of both its subsites layered with hydrophobic residues.

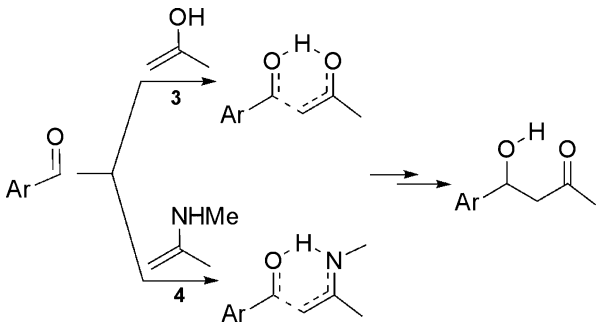
In a Hammet plot for k_{cat} and k_{unc} (see ESI†), both series of data correlate well with σ_p, with similar slopes of +2.08 (uncatalyzed) and +1.95 (catalyzed). In the addition of the acetone enolate to benzaldehydes, a higher ρ value of around +3 can be estimated for base-catalysis,[18] while the acid-catalyzed adol addition shows a moderately negative ρ value.[19] A concerted reaction such as the borane reduction occurs with a moderately positive ρ of +0.66.[20] The intermediate value of ρ (+2.08) obtained for our uncatalyzed process at pH 7.5 is consistent with a reaction occurring between neutral species with moderate charge transfer to the aldehyde in the transition state and a similar conclusion can be drawn for the BSA-catalyzed reaction (ρ +2.08). An enamine-mediated mechanism, involving a low pK_a lysine, has been inferred for aldolase antibody 24h6A from a +2 value of ρ[21] and also seems entirely compatible with our results. We have also found that incorporation of ^{18}O by acetone (10% in $H_2{}^{18}O$) is 15 times faster in the presence of 200 μM BSA. The rate of exchange is comparable to that obtained with aldolases on ketone substrates,[22] which has been postulated to proceed *via* the same enamine intermediate of the aldol reaction.

We have also carried out a series of DFT calculations on the reaction of aldehydes **1b–e** with acetone's enol **3** (Scheme 2) as a model for the uncatalyzed reaction and *N*-methylethenamine **4** as a model of the catalyzed process.[23] Both reactions prefer a concerted mechanism, *via* cyclic transition states. Formation of the C–C bond is significantly more advanced in the enamine transition state, with proton transfer lagging behind (see ESI†). A good agreement is found between experimental constants for the albumin-catalyzed reaction and calculated constants for the model enamine reaction, thus further supporting the proposed enamine mechanism for the catalyzed process (Table 2). On the contrary, the relative rate constants calculated for the enol reaction do not reproduce well the experimental values for the uncatalyzed reaction. Probably the model is an over-simplification of the actual

Table 2 Experimental relative kinetic constants for the uncatalyzed and BSA-catalyzed aldol reactions of aldehydes **1b–e** with acetone and calculated values for the reaction of the same aldehydes with acetone's enol and *N*-methylethenamine

	$k_{rel,unc}$ exp	$k_{rel,enol}$ calc[a]	$k_{rel,cat}$ exp[b]	$k_{rel,enamine}$ calc[a]
1b	1	1	1	1
1c	7.9	1	3.5	3.9
1d	50	4.8	64	26
1e	73	11.8	100	88

[a] B3LYP/6-311++G(3d,3p)/CPCM; in water at 37 °C; from the activation barriers after ZPE correction. [b] BSA.



Scheme 2 Model aldol reactions of acetone enol **3** and *N*-methylethenamine **4** *via* cyclic transition states.

mechanism for the spontaneous aldol reaction in water, which might involve discrete water molecules.[24]

The proposed enamine mechanism may also account for the switch in enantioselectivity from BSA to HSA. The binding sites of the two proteins are almost superimposable, the only difference being BSA's Lys222 and Arg199 which exchange positions in HSA.[25] If we assume that the orientation of the substrate **1a** is dictated by the binding site topology, so that the large aromatic group occupies the major hydrophobic subsite, then the active site lysine would be facing opposite sides of the carbonyl in the two proteins. Accordingly, the enamine should attack the Si face of the aldehyde in BSA and the Re face in HSA (Fig. 3).

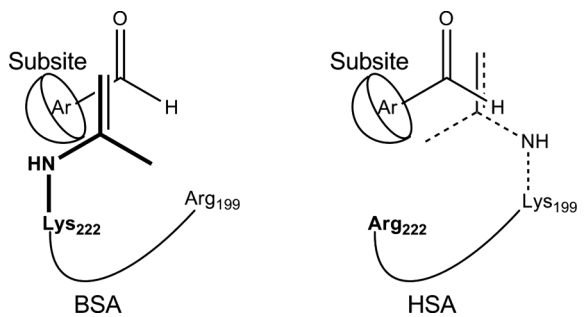


Fig. 3 Origin of the stereoselectivity switch in the enamine-mediated addition of acetone to aldehyde **1a**.

Finally, we investigated the possibility of obtaining fully functional aldolase peptides starting from the sequence of serum albumins. Single domains of HSA can be expressed in a functional form,[26] but we reasoned that shorter peptides might also be stable, provided they contain essential structural features. We have thus identified a 103 aminoacid sequence (IIa103) corresponding to

Table 3 Initial rates of formation of **2a** in the presence of proteins

	v_{obs}[a] 10^9 [mol l^{-1} s^{-1}]
No protein	0.325
MBP	0.303
IIa103-MBP	8.73
HSA	16.5
BSA	9.9

[a] 2mM **1a** and 200 μM protein in 10% acetone; 37 °C.

HSA's residues 191–294 which contains all the residues of the IIa binding site (see ESI†). This fragment corresponds approximately to half the domain II and contains all the disulfide-forming cysteines. IIa103 has been expressed in *E. Coli*, in fusion with the maltose binding protein (MBP).[27] Formation of aldol **2a** is accelerated by IIa103–MBP, its effect being comparable to that of native albumins (Table 3), while MBP alone is devoid of catalytic activity.

In addition to providing the first example of a functional albumin subdomain, this result enforces the evidence that the observed aldolase activity is not due to impurities in commercial albumin preparations. This is always a concern with albumins, but can be ruled out in this case because: a) albumins originating from two different species show similar activity, but opposite stereoselectivity; b) the catalytic activity is inhibited by warfarin, a typical albumin ligand, and irreversibly suppressed by modification of the active site lysine with fluorescein isothiocyanate; c) catalytic activity is maintained in the artificial protein IIa103-MBP obtained from bacteria. In conclusion we have shown that the aldol addition can also be included in the assortment of reactions catalyzed by serum albumins. Both bovine and human proteins catalyze the reaction with a specific enamine mechanism taking place inside the IIa binding site, and with opposite stereoselectivity. The finding that aldolase activity is preserved in the albumin-derived peptide IIa103 opens the way to the possibility of selecting new aldolase peptides with enhanced efficiency and stereoselectivity from mutated libraries.

Notes and references

‡ With commercial preparations of antibody 38C2 the reaction was considerably slower than with albumins and ee was measured at 10% conversion.

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