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A new red blood cell substitute*

Donor red blood cell is the standard replacement when hematocrit falls below the critical level, but it is not without its problems (1). Can red blood cell substitutes with the following properties fulfill some of the special needs for critical care medicine? "There is no need for time-consuming cross-matching or typing in the hospital setting and therefore this can be given immediately on the spot. There is no need for refrigeration since they can be kept at room temperature for >1 yr in the ambulance, in the field, or in the emergency room. There is no potential for infectious agents since they can be sterilized. There is no limit in the amount that can be made available." This has been a dream for many years, but as shown by the article in this issue of *Critical Care Medicine* by Dr. Sakai and colleagues (2) and by research and clinical trials from other centers on other types of blood substitutes, we are coming very close to this possibility.

The dream for a complete red blood cell substitute started in 1964 with the publication of the first attempt to prepare a complete artificial red blood cell (3). This fulfilled the previously mentioned properties except that the artificial cells did not circulate for sufficient length of time to carry out useful functions. As a result, emphasis moved from the overly ambitious attempt at complete red blood cell substitutes to less complex oxygen carriers (4–8). These included perfluorochemicals, polyhemoglobin, conjugated hemoglobin, intramolecularly crosskined hemoglobin, and recombinant human hemoglobin (4–8). Of these, polyhemoglobin is in the most advanced stage of development. Polyhemoglobin is based on the idea of using diacids (3) or glutaraldehyde (9) to cross-link hemoglobin. This basic idea has been developed independently by two groups in the form of glutaraldehyde cross-linked human polyhemoglobin that is in the final stages of phase III clinical trial (10) and glutaraldehyde cross-linked bovine polyhemoglobin that awaiting Food and Drug Administration approval (11). They published a number of articles including the results of their clinical trials (10, 11). Very briefly, they have infused up to 20 units of polyhemoglobin each time into patients during trauma surgery (10) or in other types of surgery (11). They were able by doing this to maintain hemoglobin at the required concentrations (10, 11). These polyhemoglobins can be stored at room temperature for >1 yr and can be sterilized to remove and inactivate infectious organisms or factors. These polyhemoglobins do not contain blood group antigens and thus can be used without cross-matching and typing. Their circulation half-time is >24 hrs. Unlike single hemoglobin molecules, polyhemoglobin preparations that are mostly in the form of large soluble hemoglobin complexes do not cross the intercellular endothelial junctions of the blood vessels to cause vasoconstriction. Polyhemoglobin is an oxygen carrier and does not have the complete function of red blood cells. However, this is already effective in clinical trials as an oxygen carrier for use in perioperative surgery including surgeries related to critical care medicine like trauma surgery, rupture aneurysm repair, and others (10, 11).

Oxygen carrier in solution can more easily perfuse through obstructed vessels in strokes and myocardial infarction. Also, unlike donor red blood cells, these carriers can be used on the spot without the need for cross-matching and typing in severe hemorrhagic shock. However, if the ischemia is severe and prolonged, reperfusion with oxygen-carrying fluids alone may result in ischemia-reperfusion injuries. We found one way to avoid this by cross-linking trace amounts of red blood cell enzymes to hemoglobin, forming a soluble polyhemoglobin-catalase-superoxide dismutase (polyHb-CAT-SOD) complex (12). We tested this in a global cerebral ischemia-reperfusion rat model. Unlike polyhemoglobin that contains no significant enzyme activities, polyHb-CAT-SOD causes no significant ischemia-reperfusion injuries as shown by the preservation of blood-brain barrier and the absence of brain edema (13).

With polyhemoglobin nearly possible for routine clinical use as an oxygen carrier, a number of groups are perusing the next step of the original idea of a complete artificial red blood cell (3). The original short circulation time was improved by research carried out by many groups using small lipid membrane artificial red blood cells (8, 14). The surface modification of the lipid membrane using polyethylene glycol has markedly improved the circulation time to double that of polyhemoglobin (14). As shown by their article in this issue, Dr. Sakai and colleagues (2) have moved the lipid membrane artificial red blood cells to the final stages of animal studies toward clinical trials (2). They have shown the efficacy of these cells in hemorrhagic shock. In other publications, they have studied in detail the safety and efficacy of their lipid membrane artificial red blood cells (8). Another more recent approach toward a complete red blood cell substitute is being develop by our group based on nanotechnology and biodegradable polymer to prepare nano-dimension artificial red blood cells (6, 15). This biodegradable polymer can be readily converted to water and carbon dioxide after use. The nano-dimension artificial red blood cell is a complete artificial red blood cell that contains all the red blood cell enzymes including superoxide dismutase, catalase, carbonic anhydrase, methemoglobin reductase, and others (6, 15). The circulation time is double that of polyhemoglobin (15). However, this is in a much earlier stage of animal studies compared with the lipid membrane artificial cells reported in this issue (2).

In summary, we now have two oxygen carriers in the form of polyhemoglobin that could be very close to routine perioperative use in trauma surgery (10) and other types of surgery (11). For conditions with potential for ischemia reperfu-

*See also p. 539.

Key Words: blood substitutes; oxygen carriers; polyhemoglobin; artificial red blood cells; transfusion; ischemia-reperfusion

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sion injuries, polyHb-CAT-SOD is being developed in animal studies (12, 13). The stage is therefore set for development of complete artificial red blood cells in the form of lipid membrane artificial red blood cells (2) and biodegradable polymeric membrane nano-dimension artificial red blood cells (15). However, in conditions requiring only a simple oxygen carrier (10, 11), there is no need to go to a more complex and more costly system. On the other hand, the more complex systems (2, 12, 13, 15), when ready, will have their roles in those clinical conditions where a simple oxygen carrier cannot fulfill all the requirements.

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Peptidoglycan is an important pathogenic factor of the inflammatory response in sepsis*

Sepsis is the clinical manifestation of the host-derived systemic inflammatory response resulting from invasive infection. A majority of cases of sepsis and septic shock are secondary to infections with Gram-negative bacteria. Gram-positive organisms also account for a significant proportion of cases. The inflammatory process begins at the nidus of infection, where bacteria proliferate and either invade the bloodstream or release various bacterial components, such as endotoxin, peptidoglycan, teichoic acid, and other microbial exotoxins (1). The main therapeutic intervention following sepsis is antibiotic therapy. However, even when effective antibiotics kill bacteria, they do not affect the release of these bacterial toxins (2, 3). The interaction of these microbial cellular components with macrophages, monocytes, or other host cells induces the release of inflammatory mediators that play a major role in the pathophysiology of septic shock (2, 3). This interaction of microbial products and host cells is a major determinant of the innate immune response and represents the first line of defense against pathogens by promoting acute inflammatory responses and evoking early cellular infiltration at the site of infection and tissue injury. The innate immune system has evolved a complex network of receptors, which rapidly identify pathogens based on invariant molecular structures that are shared by a variety of microorganisms. Among these invariant macromolecular structures, peptidoglycans are recognized by cells of the host through specific interactions with cell surface receptors, such as the Toll-like receptors (TLR) (4, 5).

It has been demonstrated that activation of mitogen-activated protein kinase homologs and other kinases mediates the transduction of extracellular signals from the receptor levels to the nucleus and is a pivotal event in the regulation of the transcription events that determine functional outcome in response to stress (6). This signaling cascade is rapid and enables the cells to respond to environmental changes by inducing a prompt production of proinflammatory and anti-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-10, and interferon-γ (6).

In this issue of *Critical Care Medicine* Dr. Wang and colleagues (7) show that *in vivo* administration in the rat of a purified extract of peptidoglycan of *Staphylococcus aureus* causes liver and renal dysfunction, which correlates with increased tissue gene expression and plasma elevation of cytokines. These events are associated with increased DNA binding activ-

*See also p. 546.

Key Words: sepsis; nuclear factor-κB; Toll-like receptor; signal transduction; peptidoglycan

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総　説

リポソームと補体系との相互作用

Interaction between complement system and liposomes

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和文抄録

脂質二分子膜からなるリポソームはその内部に様々な物質を包埋することができ、抗ガン剤、抗真菌剤などがリポソーム製剤として応用されている。人工酸素運搬体においても、セルフリー・ヘモグロビン修飾体に起因する副作用防止を目的とし、リポソームにヘモグロビンを包埋したセル型人工酸素運搬体の開発研究が行われている。しかし一方で、生体にとって異物であるリポソームは生体の感染防御機構により認識され、種々の生体反応を引き起こす。その一つが補体活性化であり、構成脂質や動物種に依存して古典経路あるいは第二経路が活性化される。また、リポソーム投与による補体活性化が原因と考えられる副作用が、動物試験および臨床例で報告されている。本総説では、補体系を活性化するリポソームの特徴と機序について概説する。

Abstract

Liposomes consisting of lipid bilayer can encapsulate various kinds of compounds such as anticancer drugs and antifungal antibiotics. Encapsulation of hemoglobin into liposomes as an artificial oxygen carrier has been studied to prevent adverse reactions due to cell-free hemoglobin substitutes. However, liposomes administered are recognized by host defense system, resulting in the trigger of a variety of biological reactions. Complement system is activated by liposomes *via* classical and/or alternative pathway depending on a lipid composition of liposomes and animal species used. Recently, adverse reactions caused by liposome administration have been reported in experimental animal models and human clinical settings. This review will outline the mechanisms of complement activation by liposomes and their characteristics.

Keywords

liposome, complement, artificial oxygen carrier, anaphylaxis

1．はじめに

1965年、イギリスのBanghamらは、両親媒性の性質を持つ脂質を水溶液に分散させると、物質を内包できる脂質閉鎖小胞が形成されることを発見し、後にその小胞はリポソームと呼ばれるようになった．それ以降、生体膜の研究において、リポソームは欠かすことの出来ないモデルとして広く用いられている。このモデルにより、生体膜はリン脂質二分子膜にタンパク質が埋まった構造をしているという流動モザイクモデルが広く受け入れられるようになった。そして生体膜の基礎研究を行う上で、リポソームは生体機能解析のモデル系として用いられ、相転移、相分離、物質透過に関わる流動性、膜電位、表面電位、膜透過性、膜損傷、脂質過酸化、再構成膜による膜タンパク質の機能などについて多くの知見が得られている。特に免疫学の分野では、補体の活性化要因や作用機序を解析する人工膜として貢献してきている。

2．リポソーム製剤

リポソームによる膜の基礎研究の進展とともに、内部に様々な物質を封入できることから医学分野での応用が試みられている（1, 2)。封入されるものとして、抗ガン剤、免疫賦活剤、抗菌剤、抗炎症剤、遺伝子などがある。薬剤をリポソーム製剤とする目的は、薬効を持続させるために徐放性を持たせたり、標

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的臓器に効果的に薬剤を到達させ副作用の軽減をはかることである。また遺伝子をリポソームに封入し、細胞融合により外来遺伝子を細胞に導入するという遺伝子治療への試みもなされている。使用目的に応じてリポソームの構成脂質、サイズ、構造、荷電などを変えて調製することにより、ガン細胞や貪食細胞に取り込まれやすくすることができる。また局所投与も有効な方法である。このような利点から、ドラッグデリバリーシステム（DDS）としてのリポソームの応用が考案されている（3)。また、リポソームの血中半減期を長くする研究も盛んであり、血中に長く留まり酸素運搬を担うというセル型人工酸素運搬体としての利用に期待が持たれている（4)。

3．リポソームの人工酸素運搬体への応用

セル型人工酸素運搬体創製の可能性は、1957年のDr. Changの検討により示唆された。その後1980年代、ヘモグロビンを脂質二分子膜で包埋したリポソーム包埋ヘモグロビン酸素運搬体が検討され始めた。1990年代には様々な改良が加えられ、*in vitro* および動物モデル試験により多くの知見が得られてきている。多くの酸素運搬体がセルフリー型のヘモグロビン分子修飾体であり、分子修飾により血中半減期の延長がはかられたものの、血管内皮由来弛緩因子NOの消去によると考えられる血圧上昇、消化管運動抑制などの副作用が観察された。この副作用は、ヘモグロビン分子が血管内皮のギャップジャンクションを通過する大きさであり、内皮細胞と平滑筋の間隙に入り込んだヘモグロビンがNOを消去するからであると考えられている。したがって、脂質膜によりヘモグロビンを包埋し分子サイズを大きくした人工酸素運搬体は、このような副作用の防止に有望であると考えられている。加えて、抗酸化物質などを同時封入することにより、ヘモグロビンの機能維持、酸素運搬能以外の機能の付与などに期待が持たれている。

リポソームの体内動態は、(1) 投与法、(2) 粒子径と投与量、(3) 構成脂質と荷電、表面修飾、(4) 封入物質、により異なってくる。現在、セル型人工酸素運搬体を精力的に開発研究しているグループには、早稲田大学/慶應義塾大学/北海道血液センター、テルモ社、アメリカ陸軍研究所がある。三者三様の開発を行っているが、人工酸素運搬体の投与方法は静注であり、封入物質はヘモグロビン（ストローマ・フリー・ヘモグロビン）と共通している。粒子径は、封入効率、作業性、貪食細胞との相互作用や血流動態などを考慮し、いずれも200-400 nmとなっている。また、投与量により血中半減期が異なることも明かとなっている。したがって、それぞれのグループから報告される異なる生体反応は、構成脂質と荷電、表面修飾に起因すると考えられる。つまり生体適合性を高めるためには、構成脂質と荷電、表面修飾に細心の注意を払わなければならないと言える。

4．リポソームに対する生体反応

リポソームは細胞膜と同様リン脂質を構成成分としているため、代謝産物も含めて安全かつ生体適合性に優れていると考えられている。とは言え、生体にとっては異物であるため、血球細胞や血漿タンパク質と相互作用する。またこの作用は、リポソームの大きさや構成脂質および荷電により異なってくる。リポソームは、異物や老化細胞などの処理を担う細網内皮系により血流中から排除される。また、陰性表面荷電や脂質が反応の引き金となる補体系、凝固系、カリクレイン－キニン系へも影響を及ぼすと考えられる（5, 6)。近年、リポソームによる補体系の活性化が、副作用との関連において注目されている。ブタモデル実験において、ある種のリポソームは静脈投与によりアナフィラキシー様反応、肺動脈圧の上昇、心拍出量減少、肺血管抵抗上昇など偽アレルギー反応が起こり、これらの症状と補体活性化との関連が指摘されている（7-9)。したがって、セル型人工酸素運搬体として投与されるリポソームは、補体系を活性化しないことが重要となる。

5．補体とは

ここでは概要を紹介するので、詳しくは成書を参照されたい(10)。19世紀末、血清中に非耐熱性の溶菌作用を示す因子の存在が発見され、これが後に補体と呼ばれることとなった。補体系は30種以上の因子からなり、生体にとっての異物、例えば細菌のリポ多糖などに反応する自然免疫として殺菌、溶菌を担い、また獲得免疫の補助として、生体の感染防御機構において重要な役割を果たしている。補体が活性化される経路には、古典経路（classical pathway）と第二経路（alternative pathway)、レクチン経路の3つがあり、プロテアーゼカスケードを成している（Figure 1)。それぞれの経路の活性化は異なる機序により起こる。

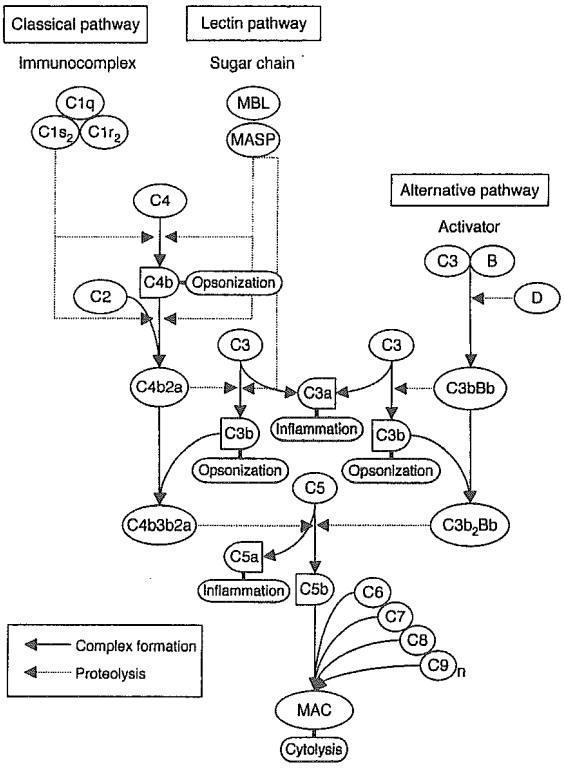

Figure 1. Overview of complement activation pathway

古典経路の活性化は抗原に結合した抗体が構造変化を起こし、そのFc部分に補体成分C1が結合することから始まる。活性化のためにはC1のサブユニットであるC1qがFcに多価性に結合する必要があることから、抗体がIgMであれば1分子でよいが、IgGでは少なくとも2分子以上が近接して抗原に結合しなければC1qの多価性結合が起こらず補体活性化は起こらない。こうして活性化したC1はC4をC4aとC4bに加水分解し、抗体結合部位周辺の細胞膜へのC4bの沈着が起こる。この沈着は、加水分解により表出したC4bのチオエステル結合（-S-CO-）が、膜表面のNH_2基、OH基と共有結合する事により起こる。その後C4bはC2aと複合体（C4b2a）を形成してC3分解酵素活性を獲得し、C3を活性化（C3aとC3bに分解）する。C3活性化は古典経路、第二経路、レクチン経路に共通した反応であり、これ以降のカスケード反応は共通である。

レクチン経路は血清中に存在するマンノース結合レクチン（MBL）/MASP-1& 2複合体を介する経路である。MBLの糖鎖認識ドメインが細菌やウイルス表面のマンノースやN-アセチルグルコサミンと結合するとセリンプロテアーゼ前駆体であるMASPsは活性化し、C4をC4aとC4bに、C3をC3aとC3bに加水分解する。こうして抗体非依存的に補体の活性化が起こる。

第二経路では特異的な認識を必要とせず、補体活性化が起こる。自然状態ではC3の活性化が僅かずつではあるが持続的に発生しており、血中のH因子、I因子という抑制因子によりそれ以降のカスケード反応が抑え込まれている。この活性化が細菌表面などで起こるとC3bの沈着が始まり、抑制因子による阻害効果が十分働かなくなり、一連の活性化カスケード反応が進行する。

それぞれの経路で生じたC4bおよびC3bは複合体C4b3b2aおよびC3b2Bbを形成しC5をC5aとC5bに加水分解する。生じたC5bは次々と補体C6、C7、C8、C9と複合体を形成し、膜傷害複合体（membrane attack complex：MAC）となる。MACは細胞膜を貫通する穴を形成し、膜内外のイオンバランスを崩すことで膜破壊を引き起こし、殺菌作用を示す。一方、細菌やウイルスに結合したC4b、C3bは目印となり（オプソニン効果）、補体レセプターを有する食細胞による貪食作用を増強する。もう一つの補体の生理活性として、加水分解で生じたC3a、C5a、(C4a)、の炎症メディエーターとしての作用がある。アナフィラトキシンと呼ばれるこれらのペプチドは、肥満細胞からのヒスタミン遊離、血管透過性の亢進、平滑筋の収縮などを引き起こし、局所に炎症を引き起こす。また走化性因子として炎症部位に好中球や単球・マクロファージを引き寄せ、活性化する。

したがって、リポソームにより補体系が活性化されると、リポソームの破壊やアナフィラトキシンによる全身性の炎症反応が起こり、重篤な副作用を引き起こす可能性があることから、セル型人工酸素運搬体には高い生体適合性が求められる。

6．リポソームと補体の反応

リポソームと補体系との相互作用に関しては、研究者によって相反する結果が報告されている。この矛盾は、リポソームの表面荷電密度、粒子径、膜流動性、構成脂質、脂質飽和度、血清あるいは血漿に加える脂質量、動物種の違いによりもたらされる（11）。そのため現時点では、リポソームが補体系に及ぼす影響を、個々の結果に基づき系統立てて解説することは困難である。以下にこれまでの主だった検討結果をまとめた。一般的には、中性荷電脂質を構成成分としコレステロール含量が低い場合には、多くの動物種で補体活性化がみられない傾向にあるようだが、相矛盾する結果もあり、相互作用の複雑さが伺われる。

6.1　第二経路の活性化

血清あるいは血漿に酵母や細菌、異種赤血球を添加すると補体系が活性化し、これらは抗体に依存しない第二経路によることが知られていた。その機序を探るため、リポソームに膜タンパク質を埋め込んで人工膜モデルを構築し、検討が進められた。シアル酸糖タンパク質で表面をコーティングすると第二経路による補体活性化が抑制され（12）、また血中滞留時間を延ばすことが出来る（13）。その一方、未修飾のリポソームを血清と反応させると内容物が漏出し、またリポソーム表面がオプソニン化されることから血中からの消失が促進され、DDSとしての使用が問題になることが明らかになり始めた。

リポソーム表面荷電の影響

多くの検討において第二経路依存性は、Ca^{2+}非依存的Mg^{2+}依存的な補体の活性化により古典経路と区別している。また活性化の有無は、リポソームへのC3（C3b、iC3b）の結合、感作赤血球溶血活性の低下などにより判断している。リポソームによる第二経路活性化は表面荷電に依存すること、即ちstearylamineに付与される陽性荷電が活性化の引き金になり、また、脂肪酸鎖長や飽和度も影響する（14, 15）。モルモットおよびヒト血清を用いた検討では、陰性荷電リポソームでは第二経路は活性化されない（15）。一方、ラット血清においては、陽性荷電、陰性荷電両リポソームとも古典経路により補体活性化を引き起こすと報告されている（16）。また、ラット血漿を用いた検討では、陰性荷電リポソームにより古典経路ではなく第二経路により補体が活性化されるとの報告もある（17）。この乖離は先の論文の著者らにより、血漿分離に用いたヘパリンが第二経路活性化を優位にしているためであろうと指摘されている（16）。リポソーム製剤が生体に投与されたときに相互作用するのは血漿なので、血漿を用いた検討の方がより臨床に近い状態といえる。しかし、*in vitro* の検討においては、用いる抗凝固剤によっては補体系に影響を及ぼすため、注意が必要と思われる。

コレステロール含量の影響

多くのリポソーム製剤はphosphatidylcholine（PC）とcholesterol（CHOL）を主たる構成脂質としている。CHOLは膜の流動性を高めるために用いられ、PC:CHOL（55:45）では表面荷電は中性であり、モルモット,ラット、およびヒト血清

Table 1. Electric charge of liposomes and the activation of complement system

Charge	Lipid composition	Subject	Classical pathway	Alternative pathway	Reference
Positive	DMPC:CHOL:GC:SA	Human serum	−	+	13
	PC:CHOL:SA	Human serum Guinea pig serum	− −	+ +	14
	PC:CHOL:SA	Rat serum	+	−	15
Negative	DMPC:CHOL:GC:DCP	Human serum	−	−	13
	PC:CHOL:(PG/PA/CL/PI/PS)	Human serum Guinea pig serum	+ +	− −	14
	PC:CHOL:(PG/PA/CL/PI/PS)	Rat serum	+	−	15
	HEPC:CHOL:DCP	Rat plasma	−	+	16
	PC:CL/PG PC:PA	Human purified complement	+ −	ND ND	32
	PC:CHOL:CL/PG/PI	Human Ig-depleted serum	+	ND	33
Neutral	PC:CHOL PC:CHOL:DPPE	Human serum Guinea pig serum Rat serum	−	−	14, 15
	PC:CHOL PC:CHOL:DPPE	Human serum	ND ND	− +	17

+, Activated; -, Not activated; ND, Not determined.
DMPC, dimyristoyl phosphatidylcholine; CHOL, cholesterol; GC, galactosyl ceramide; SA, stearylamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; HEPC, hydrogenated egg PC; DCP, dicetylphosphate; DPPE, dipalmitoyl phosphatidylethanolamine.

Table 2. Cholesterol contents and the activation of complement system

Lipid composition	CHOL(Mol %)	Subject	Classical pathway	Alternative pathway	Reference
DMPC:CHOL:GC:SA	20 43, 60	Human serum	− −	− +	13
PC:CHOL:PG (lipid 10 mM)	0 - 10 25 - 40	Rat serum	− +	− −	15
PC:CHOL	45	Human serum Guinea pig serum Rat serum	−	−	14, 15
HEPC:CHOL:DCP	22, 33 44	Rat serum	+ −	− +	18

+, Activated; -, Not activated

では補体活性化は起こらない（15, 16）。これはdipalmitoyl phosphatidylethanolamin（DPPE）を含むリポソームPC:CHO:DPPE（35:45:20）においても同様であった（15, 16）。その一方、PC:CHOL（66:33）では活性化が起こらないがDPPEを導入することによりDPPE含量依存的に第二経路の活性化が起こり、これはアミノ基をジメチル化した三級アミンおよびトリメチル化したPCにするとその活性が失われるとの報告もある（18）。両者の違いは用いたPCによる膜の流動性の違いによるかも知れないと推測されている。話はCHOLに戻るが、ヒト血清において高CHOL含量（43 mol%）により第二経路を介した補体の活性化が起こるとの報告もある（14）。また、ラットにおいては高CHOL含量（44 mol%）では第二経路を介した活性化が、低CHOL含量（22 or 33 mol%）では古典経路を介した活性化が起こり、古典経路の活性化は血液中に存在する抗脂質自然抗体、特にIgMが関与しているとの報告もある（19）。

抗体依存性の第二経路活性化

基本的に第二経路の活性化には抗体は必要としないが、抗体介在による第二経路の活性化も報告されている（20）。C4欠損モルモット血清（C4D-GPS）とparagloboside（PaGlo）含有リポソームを反応させるとCa^{2+}非依存性に補体活性化が起こる。しかしspecific pathogen-free飼育モルモット血清（SPF-GPS）では補体活性化が起こらない。また通常飼育モルモット血清（GPS）によるPaGloリポソーム溶解はPaGlo抗体量と相関すること、Ca^{2+}非存在下でSPF-GPSに非働化C4D-GPSあるいはC4D-GPSの精製IgM画分を添加するとPaGloリポソームを溶解することが観察された。これらのことから、PaGloリポソームは、通常飼育ラット血液中のPaGloに対するIgMタイプ自然抗体を介した第二経路の活性化により溶解したと結論づけられた。こうした自然抗体は自己の抗原とも反応し補体活性化を引き起こす可能性があるが、生体膜ではシアロ糖タンパク質の共

存によりその反応が抑制され、それにより非自己に対してのみの反応性を発揮しているとの仮説が提案されている（21）。

6.2 古典経路の活性化

古典経路による補体系活性化とリポソーム傷害の機序解明には、ヒツジ赤血球膜により調製したリポソームと、抗ヒツジ赤血球ウサギ血清およびモルモット血清を用い検討された。これはヒツジ赤血球の糖脂質画分に見いだされたForssman抗原と抗血清の抗原抗体反応がリポソーム上で起こり、そこに補体が関与して内容物の漏出を引き起こす（22, 23）。補体タンパク質のアッセンブリーの検討は主にヒツジ赤血球を用い行われていたが（24）、構成脂質の影響をみるため、ヒツジ赤血球膜脂質に種々の脂質を添加したリポソームでの検討が行われた。この結果、第二経路と同様、古典経路によるリポソーム傷害にも脂肪酸鎖長や飽和度が関与し、鎖長が長くなると補体による内容物の漏出が低下し（25）、補体の活性化が抑制される（16）。これは脂質二分子膜が厚くなることによると思われる。補体活性化とリポソーム内容物の漏出はコレステロール含量の増加および不飽和脂肪酸の存在によっても抑制される（16, 25）。

コレステロールと自然抗体

全ての動物は血液型抗体に代表される、様々な生体物質に対する自然抗体を持っている。リポソーム研究においては種々の脂質に対する自然抗体が報告されている（20, 26）。ウサギ血清中にはスフィンゴミエリン含有リポソームと反応する自然抗体が存在し、モルモット血清存在下で内容物の漏出を誘導し、この反応はIgM依存性であった（26）。高CHOLリポソーム（71%）はヒト血清により傷害を受け、内容物の漏出が起こるが（低CHOL（43%）およびモルモット血清では起こらない）、これは古典経路を介した補体活性化によるものと考えられていた（27）。後に、この補体活性化はヒト血清中に存在する抗CHOL自然抗体により誘導され、陽性荷電リポソームやeggPCをDPPCで置き換えたリポソームでは活性化しないことが報告された（28）。また検討したヒト血清のほぼ全てにおいて、抗CHOLIgGおよびIgMが検出されている（29）。近年、この抗CHOL自然抗体が血中LDLコレステロールの代謝に重要な役割を果たしているとの説が提唱され（30）、このことはアテローム性動脈硬化症と関連があるのかも知れない（31）。またヒト血清中には交差反応性抗リン脂質自然抗体の存在も指摘されており、リポソームへの結合はPCやATPで抑制されること、リポソームとの混合により補体消費が起こることが報告されている（32）。

抗体非依存性の古典経路活性化-陰性荷電リポソーム

抗体依存性の第二経路の活性化が示されているのと同様、抗体非依存性に古典経路が活性化されるとの報告も数多い。陰性荷電を持つcardiolipin（CL）およびPGからなるリポソーム（PC:CL（20:80）、PC:PG（20:80））には直接C1が結合して活性化し、この作用はCLでより強い（33）。また、抗体依存性の活性化の場合、生理的なC1インヒビターの結合はC1活性化を阻害しないが、PC:CLリポソームではCL含量依存性（CL:40-80%）にC1インヒビターにより活性化が抑制される。一方、PC:PGではPG:40-80%の範囲でC1インヒビターにより活性化が抑制される。C1qはPC:CLのCL含量依存性に結合したが、PC:PGには結合しなかった。CLによる古典経路の活性化は抗原抗体複合体のそれと機序が異なり、またCLとPGでも異なる。リポソームによる抗体非依存性の古典経路の活性化には、マイクロモル濃度という比較的多量の脂質を必要とする。種々の陰性荷電リポソームにより補体活性化が起こるが、化学構造、荷電密度により活性化の度合いが異なる（34）。陰性荷電リポソームによる第二経路の活性化は、ヒト血清においてはみられていない（34）。

6.3 細網内皮系とリポソーム

リポソームによる補体活性化の結果、リポソームはオプソニン化される。オプソニン化され表面にC3bを共有結合した異物は補体レセプターCR1、CR3を介して細網内皮系により排除されるが（35）、リポソームもオプソニン化により肝臓のKupffer細胞により貪食される（36）。この貪食はリポソームのサイズに依存し、eggPC:CHOL:DCP（50:40:10）リポソームでは、粒子径200 nmでは補体活性化が起こらず、ラット肝の取り込みもオプソニン未処理リポソームと変わらないが、粒子径800 nmでは肝臓の取り込みも増大し、補体も活性化する（37）。しかし後に、補体の活性化は粒子の大きさではなく粒子の数に依存し、粒子径200 nmのリポソームでも400 nmおよび800 nmのリポソームと同等の内容物漏出が起こることが報告された（38）。表面荷電に関しては、マウス腹腔マクロファージもオプソニン化リポソームを貪食するが、リポソームの陰性荷電の増大とともに貪食量が低下するとの報告がある（39）。つまりこの報告によると,リポソームの陰性荷電は、*in vitro*では抗体非依存性に古典経路を活性化してオプソニン化を促進するが、マクロファージによる貪食を抑制することになる。また、CHOL含量はその増大とともにラット腹腔マクロファージへの付着と取り込みが増大するが、さらに取り込み速度にも影響を及ぼし、補体依存性と非依存性の貪食経路の存在が示唆されている（40）。加えて、動物種によっては*in vitro*と*in vitro* の結果の乖離にも注意を要する（41）。ラットでは血小板上に補体レセプターが存在するため、投与されたPG含有リポソームはオプソニン化されて血小板と結合し、血小板減少を来す（42, 43）。一方、ヒトでは血小板には補体レセプターCR1が存在しないため、PG含有の有無にかかわらず補体を活性化しオプソニン化されたリポソームとは相互作用しないと考えられる（43）。

6.4 補体からのエスケープ

このようにオプソニン化されたリポソームは細網内皮系により捕捉され、血中から速やかに消失してしまう。そのため、補体からのエスケープおよび血中半減期の延長を目的として、リポソームの表面修飾が検討されてきた。リポソーム構成脂質にmonosialoganglioside（GM_1）を用いるとマウスにおいて血中

滞留時間が延長することが示された（13)。しかし、マウスでは血中滞留時間が延長したものの、ラットにおいては逆に短縮された（44)。このリポソームをヒト血清で処理したのち肝灌流実験に供しても、マウス肝では延長するもののラット肝では血中滞留時間は短縮した。一方、リポソームをポリエチレングリコール（PEG）修飾すると、ヒト血清との反応においても内容物の漏出は認められず、マウスにおいて血中半減期は30分以下から5時間に延長した（45)。ラットにおいてもこの特性は維持され、コレステロールや陰性荷電脂質の影響を受けなかった（46)。補体活性化の防止には、PEG分子量が大きい場合は、より少ない割合でリポソームに導入しても効果がある（47)。

しかし、PEG修飾リポソーム製剤において、予想外な副作用が報告され始めている（9)。抗ガン剤doxorubicinを封入したPEG修飾リポソーム製剤Doxil/Caelyx（hydrogenated soy PC:CHOL:PEG2000-distearoyl PE（60:20:20））は1995年にエイズ関連カポジ肉腫を適用として認められた。しかし、Doxilの投与により急性症状として、呼吸困難、紅斑、顔面紅潮、胸痛、背面痛、血圧変化が報告されている。Doxilより一足早く認可された深在性（内臓性）真菌感染治療薬AmBisome（amphotericin B封入非PEG修飾リポソーム製剤）でも、輸注後数分以内の急性副作用（血圧低下、紅斑、発熱、気管支痙攣、顔面浮腫）が観察され、原因は内包された薬剤ではなくリポソームに起因するアナフィラキシー反応と考えられている（48, 49)。また、Doxilと同じ脂質組成である放射性画像診断造影剤^{99m}Tc標識PEGリポソームの投与でも、紅潮や胸部圧迫感の発症が見られ（50)、0.1 μmol/kg bodyweightの少量投与では4時間後のシンチグラムで肝臓、脾臓、骨髄に高い放射活性が検出された（51)。このことは、^{99m}Tc標識PEGリポソームが血中から細網内皮系に取り込まれたことを示す。^{99m}Tc標識PEGリポソーム投与により副作用を起こした患者において輸注直前、副作用発症直後の血液を調べたところ、C3、BおよびC4の低下が認められ、補体の活性化が示唆されている（52)。PEG修飾されているにもかかわらず補体が活性化される理由は不明であるが、PGを構成脂質として用いているためかもしれない。

7．セル型人工酸素運搬体と補体

上述のように、リポソームと補体系の相互作用は、表面荷電密度、粒子径、膜流動性、構成脂質、脂質飽和度、血清あるいは血漿との混合比により左右され、また用いる動物種の違いによっても結果は様々である。これらのパラメーターに留意しながら、セル型人工酸素運搬体の開発を行うことが重要である。

アメリカ陸軍研究所のセル型人工酸素運搬体はLEH（liposome-encapsulated hemoglobin）と呼ばれ、distearoyl phosphatidylcholine（DSPC）：dimyristoyl phosphatidylglycerol（DMPG）：CHOL：α-tocopherol（50:4.5:45:0.5 mol%）からなる平均粒子径388 ± 55 nmのPEG未修飾リポソームである（53)。初期の動物実験では、ラット投与10分後には血漿の補体溶血活性（CH50）は著明に減少し、同時にトロンボキサンB_2（TXB_2）の劇的な増加が観察された。ラット血液中にはLEHに反応する抗体が検出されたが、補体活性化は第二経路を介して起こったと考えられた（53)。ラット血清を用いた*in vitro*の検討でもCH50は同様に低下し（54)、投与試験では平均動脈圧と心拍数の増加、血小板減少、血圧低下、頻脈、心係数低下などが観察された（55, 56)。血液生化学的パラメーターの変化は一過性であり、肝臓、脾臓マクロファージへのLEHの蓄積が観察されている（57)。その後、LEHに反応する抗リン脂質自然抗体がヒト血清中に見いだされ（32)、LEHは古典経路および第二経路の両経路を介してヒト補体系を活性化することが報告された（58)。またこの活性化は、可溶性補体レセプターtype 1（sCR1）により効果的に抑えることができる。このLEH投与により起こる症状は感作を必要とせず、初回投与後1-5分以内に起こることから“補体活性化関連偽アレルギー”と名付けられ、感受性の高いブタを用い、心臓脈管系への影響が詳細に調べられた。その結果、ラットと同様、平均動脈圧と心拍数の増加、血小板減少、TXB_2の産生などが観察された。このようなことから、外傷患者にとっては症状を悪化させる可能性が示唆されている（59)。

テルモ社が開発したセル型人工酸素運搬体はNeo Red Cell（NRC）と呼ばれ、hydrogenated soy PC:CHOL:myristic acid:α-tocopherol:PEG5000-PE（7:7:2:0.28:0.4）を構成脂質とする平均粒子径220 nmのPEG修飾リポソームである（60, 61)。ヒト新鮮血漿との相互作用において、生食群に比べC3a値の若干の上昇がみられたもののC5aの生成は両者ともほとんど無く、補体系への影響は無いと考えられた（62)。

早稲田大学が開発したセル型人工酸素運搬体はヘモグロビン小胞体（HbV; hemoglobin vesicles）と呼ばれ、DPPC:CHOL:DP-L-glutamate-N-succinic acid（DPEA）:PEG5000-DSPE（5:5:1:0.033）を構成脂質とする平均粒子径250 nmのPEG修飾リポソームである（63)。我々の検討の結果、HbVとヒト血清との混合において補体価CH50の低下、即ち補体の活性化は起こらないことが観察された（論文作成中)。ラット血清およびラット*in vivo*投与においても補体は活性化されない（64)。加えて、凝固系、カリクレイン－キニン系への影響も無いことが示されている（65)。HbVのゼータ電位を測定したところ、生理的食塩水中では表面荷電は-2.6 mVとほぼ中性であった。一方、構成脂質にDPEAではなくDPPGを持つPEG修飾リポソームの表面荷電も-3.4 mVであったが、凝固系に影響を及ぼし、カリクレイン－キニン系も活性化した（65)。このことは、単に表面荷電だけではなく、構成脂質の親水性頭部（head group）が影響を及ぼすものと推測される。このようにHbVは補体系を活性化しないことから、“補体活性化関連偽アレルギー反応”と呼ばれる投与直後の心血管系の急性副作用に対する懸念はほとんど無いと考えられる。またHbVのラット投与において15%投与より25%投与の方が血中半減期は15時間から24時間と長くなり、最終的にHbVは肝臓、脾臓、骨髄に分布した（66)。HbVは補体を活性化しないため、オプソニン化されないと考えられる。したがって、このような細網内皮系への分布は、補体レセプターを介さない貪食や補体以外の血漿タ

ンパク質の関与が推測される（67)。投与量増加に伴う血中半減期の延長は、個々のリポソームへの血漿タンパク質の結合量が減少したためとも考えられるが（68)、細網内皮系の飽和による可能性もある。

8．おわりに

リポソーム製剤が上市され、その使用拡大とともに補体活性化が関与すると考えられる副作用が報告され始めている。これらアナフィラキシー様の副作用は少量投与でも発生することから、人工酸素運搬体のように出血時の大量投与を想定した場合、なおさら懸念されるべき問題点である。セルフリー型人工酸素運搬体の欠点を補うべくして開発されているセル型人工酸素運搬体において、セル型故の問題点が浮き彫りになってきたといえる。リポソームによる補体活性化の機序が数多く報告され、また矛盾する報告もあることから、それらの情報を基に補体活性化を回避するリポソームを創製することは非常に困難と言える。しかし、早稲田大学が開発したセル型人工酸素運搬体HbVは、我々がその生体適合性評価を行った結果、ヒト血清と20% v/vおよび40%v/v混合しても補体系を活性化しない。このことは臨床において非常に重要な特性といえる。このHbVの脂質組成は人工酸素運搬体のみならず、現行のリポソーム製剤に応用することにより、補体関連の副作用防止にも役立つものと期待が持てる。

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Esterase-Like Activity of Serum Albumin: Characterization of Its Structural Chemistry Using p-Nitrophenyl Esters as Substrates

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Purpose. To elucidate the catalytic mechanism of the esterase-like activity of serum albumin (SA), the reactivity of SA from six species was investigated using *p*-nitrophenyl esters as model substrates.
Methods. The effect of pH and the energetic and thermodynamic profiles of SA were determined for all species for *p*-nitrophenyl acetate (PNPA). Then, kinetic and thermodynamic studies using a series of *p*- and *o*-nitrophenyl esters with different side chains and human SA (HSA) were carried out. The influence of deuterium oxide was also evaluated. Finally, the information gained was used to construct a computer model of the structural chemistry of the reaction.
Results. The pH profiles suggest that the nucleophilic character of the catalytic residue (Tyr-411 in the case of HSA) is essential for activity. This k_{cat}-dependent activity was found to increase with a decrease in the activation free energy change (ΔG). Hence, the magnitude of ΔG, which is dependent on activation entropy change (ΔS), as calculated from the thermodynamic analysis, can be regarded as an indicator of hydrolytic activity. It indicates that *p*-nitrophenyl propionate (PNPP) is the best substrate by evaluating the reactions of nitrophenyl esters with HSA. The findings here indicate that deuterium oxide has no significant effect on the rate of hydrolysis of PNPA by HSA.
Conclusions. The results are consistent with a scenario in which HSA becomes acylated due to a nucleophilic attack by Tyr-411 on the substrate and then is deacylated by general acid or base catalysis with the participation of water.

KEY WORDS: esterase-like activity; *p*-nitrophenyl esters; serum albumin; species difference; structure–activity relationship.

INTRODUCTION

As the most abundant soluble protein in the body (about 4% in serum) and the most prominent protein in plasma, serum albumin (SA) is responsible for a multiplicity of functions, including the maintenance of blood osmolarity, acting as an antioxidant, and serving as a solubilizing agent and carrier for many endogenous and exogenous compounds (1). It is also well-known that it is a major binding protein for the transport of a large number of drugs (1).

Studies of SA have also revealed the important role of this protein as a catalyst for the hydrolysis of various compounds, such as esters, amides, and phosphates (2,3). Ikeda et al. reported that the most prominent catalytic, esterase-like, active sites of human serum albumin (HSA) are closely related to its drug binding sites, because various drugs inhibit this activity (4). Thus, the active site of HSA with respect to *p*-nitrophenyl acetate (PNPA) is thought to be the same as the binding site for several benzodiazepines (site II), and the enzymatic active site with respect to nitroaspirins is in close proximity to the warfarin binding site (site I) (4). However, in spite of the extensive literature on these reactive sites, their properties and their differences among species are not known in detail.

Typically, the esterase activity (e.g., carboxylesterase activity) is more pronounced in plasma from primates than in other animals such as dogs, rabbits, snakes, and fish (5,6). The esterase-like activity of SA has been reported to be highest in human and is completely absent in SA from horse (Order Perissodactyla) (1,7). The primary structure of several species of albumin is now known (1), and mammalian types show amino acid sequence identities of about 70–80%. Therefore, it is conceivable that they have comparable three-dimensional structures. In fact, X-ray diffraction studies by Ho et al. showed that the crystal structure of horse albumin is similar to the three-dimensional structures of HSA (8,9). Studies involving site-directed mutagenesis have shown that Arg-410 and, especially, Tyr-411 are important for the esterase-like activity of HSA (10). However, many albumin species, including horse albumin, also contain arginine and tyrosine in the same or corresponding positions, but have different enzymatic activities (1). Therefore, other, and presently unknown, factors must be important as well.

In the current study, we report on a detailed examination of the esterase-like activity of albumin and the mechanism of the catalytic reaction. Species differences were first examined. This involved a determination of pH profiles and the collection of thermodynamic data on the esterase activity of SA from human, bovine, dog, rabbit, rat, and horse using PNPA as a substrate. To characterize the structural chemistry of the active site, hydrolytic reactions of a series of *p*-nitrophenyl and *o*-nitrophenyl esters with HSA were also investigated. Finally, the optimum structure of the substrate–HSA complex was constructed using the X-ray structure of the HSA:myristate (MYR):*tri*-iodobenzoic acid (TIB) complex.

MATERIALS AND METHODS

Materials

HSA was a gift from the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan), and dog, rabbit, bovine, horse, and rat serum albumin samples were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). All of the albumin samples were defatted according to Chen's method before use (11).

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ABBREVIATIONS: Arg, arginine; HSA, human serum albumin; MD, molecular dynamics; MYR, myristate; PNPA, *p*-nitrophenyl acetate; PNPE, *p*-nitrophenyl esters; PNPP, *p*-nitrophenyl propionate; SA, serum albumin; TIB, *tri*-iodobenzoic acid; Tyr, tyrosine.

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PNPA, *p*-nitrophenol, *o*-nitrophenyl acetate, and *o*-nitrophenol were purchased from Nacalai Tesque (Kyoto, Japan), and other *p*-nitrophenyl esters were ordered from Sigma Chemical Co. Deuterium oxide (D > 99.75%) was obtained from Merck Co. (Darmstadt, Germany). All other chemicals were of analytical grade.

Procedures for Kinetic Runs

The buffer systems used were as follows: pH 6.0–8.5, 1/15 M phosphate; pH 9.0–10.5, 1/20 M borate. The ionic strength of each buffer was not fixed, because the addition of NaCl to the buffer decreased the reaction rates in a complicated manner (12). The reaction medium contained 0.5% (v/v) acetonitrile. The temperature was 25°C except for the thermodynamic analysis, which was done in a range of 15~35°C.

Hydrolysis of substrate (5 μM) by various species of albumin (at least a 5-fold excess concentration over the substrate) was carried out in such a way that complications by any multiple reactive sites of albumin were avoided. It is said that the substrates preferentially reacted with the primary reactive site of albumin under these conditions (12,13). The reactions were followed with a stopped-flow spectrophotometer (Otsuka Electronics Co. Ltd., Osaka, Japan) at 400 nm by monitoring the appearance of the corresponding phenol. Under these conditions, pseudo-first-order rate constants could be obtained.

Measurement of Reaction Parameters

The reactions of the substrates with albumin are assumed to proceed through the pathway shown in Fig. 1 (4). In this chart, PNPE is the substrate and EST–Alb denotes the Michaelis–Menten type complex between EST and albumin. Acyl-Alb denotes albumin acylated by the substrate, and K_S represents the dissociation constant of the complex. The rate constants of EST–Alb are represented by k_{cat} and k_0, respectively. k is the rate constant for the dissociation of Acyl-Alb.

The pseudo-first-order rate constant for the release of phenol, k_{obs}, can be represented as follows:

$$k_{obs} = \frac{k_0 K_S + k_{cat}[\text{albumin}]}{K_S + [\text{albumin}]} \quad (1)$$

Here, [albumin] is the concentration of albumin. Because the value of k_{cat} is much larger than that of k_0 (2000- to 6000-fold), so k_0 can be ignored (4). The K_S and k_{cat} values can be calculated from the intercept and slope of a double-reciprocal plot using Eq. (2):

$$\frac{1}{k_{obs}} = \frac{K_S}{k_{cat}[\text{albumin}]} + \frac{1}{k_{cat}} \quad (2)$$

Determination of pK_a

Assuming that the hydrolytic reaction catalyzed by albumin is dependent on the ionic form of Tyr-411, the rate of the reaction should be both the ionization constant (K_a) and pH-dependent, as described in Eq. (3):

$$\left(\frac{k_{cat}}{K_S}\right)_H = \left(\frac{k_{cat}}{K_S}\right) K_a \times \frac{1}{K_a + [H^+]} \quad (3)$$

Equation (3) has the same form as the Michaelis–Menten equation, where k_{cat}/K_S is the value at completely ionized Tyr form, and $(k_{cat}/K_S)_H$ is the measured value at a given $[H^+]$. K_a can be calculated as follows, where $[H^+]$ is the hydrogen ion concentration.

$$\left(\frac{k_{cat}}{K_S}\right)_H = \left(\frac{k_{cat}}{K_S}\right) - \left(\frac{k_{cat}}{K_S}\right)_H [H^+] \times \frac{1}{K_a} \quad (4)$$

The pK_a values were obtained using least squares method.

Thermodynamic Analysis

The free-energy change for an enzymatic reaction has generally been discussed according to the literature (14). Each free-energy parameter was calculated using Eqs. (5), (6), and (7), where ΔG_S is the free-energy change for the initial reaction of the enzyme and substrate, ΔG the activation free energy for the rate-determining step, ΔG_T the free-energy difference for the reaction, R the gas constant, T the absolute temperature, k_B the Boltzmann constant, and h the Planck constant.

$$\Delta G_S = -RT\ln(1/K_S) \quad (5)$$

$$\Delta G = RT\{\ln(k_B T/h) - \ln k_{cat}\} \quad (6)$$

$$\Delta G_T = \Delta G - \Delta G_S \quad (7)$$

The thermodynamic investigation of the albumin-catalyzed reaction was performed in the temperature range 15~35°C. The activation energy (E_a) was obtained from an Arrhenius plot of the rate constant vs. temperature following Eq. (8).

$$\frac{d \ln k_{cat}}{d(1/T)} = \frac{E_a}{R} \quad (8)$$

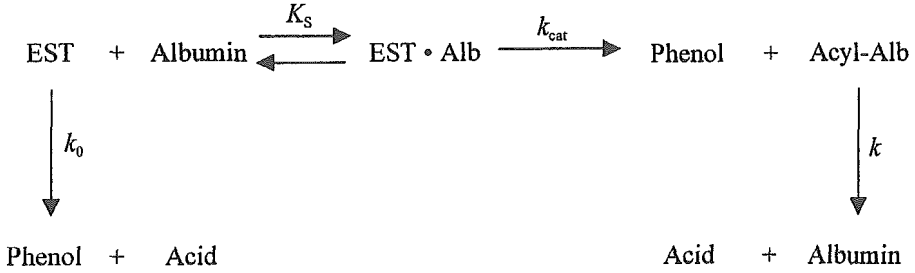

Fig. 1. Spontaneous and albumin (Alb) catalyzed hydrolysis of *p*-nitrophenyl esters (EST).

The activation enthalpy change (ΔH) and the activation entropy change (ΔS) were calculated using Eqs. (9) and (10), respectively.

$$\Delta H = E_a - RT \quad (9)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (10)$$

Conformational Analysis of PNPP

The conformational analysis of PNPP was performed using the CAMDAS 2.1β2 program (15). Ten molecular dynamics (MD) calculations were simultaneously performed using different initial structures. Each of the MD calculations was carried out for 600 ps with an integral time step of 1 fs. The lengths of the covalent bonds were fixed. The temperature of the system was maintained at 900 K in order to enhance the sampling efficiency. The Tripos force field (16) was used to evaluate the potential energy surface of the molecule. The electrostatic and hydrogen bonding term of the potential were ignored in order to avoid intramolecular hydrogen bonding. The angle and torsional terms of the potential were reduced by a factor of 0.8 in order to increase the flexibility of the molecule. Conformers were sampled at 100 step intervals, thus producing 6000 conformations for each MD calculation. A total of 60,000 conformations were preclustered with an root mean square deviation (rmsd) threshold of 0.2 Å. Superposition and rmsd calculations were performed for all of the heavy atoms. After sampling, the reclustering of the sampled conformers was performed with an rmsd threshold of 0.2 Å. Before the clustering, each conformer was minimized until the root mean square of the potential energy gradients was below 0.005 kcal mol^{-1} $Å^{-1}$. Finally, 723 conformers were obtained.

Preparation of HSA Template

To obtain the HSA template structure for docking, we used the crystal structure of the HSA:myristate (MYR):*tri*-iodobenzoic acid (TIB) complex (PDB ID code: 1bke) (17). Due to the lack of *N*-terminal residues from the 1st to the 3rd and of the *C*-terminal residue of the 585th in the PDB structure, we treated the 4th residue as the *N*-terminus and the 584th residue as the *C*-terminus. The initial positions of the other missing atoms in the PDB structure were generated using the SYBYL 6.6.2 program package (18). The Tripos force field was used. The AMBER 1991 charges (19) were used as the charges for HSA. Gasteiger-Hückel (20–23) charges were used as the charges for MYR and for TIB. Only the missing atoms were minimized using SYBYL to below 0.05 kcal mol^{-1} $Å^{-1}$ for the root mean square of the gradients. The cut-off distance for nonbonded interactions was 8 Å. Finally, the MYR and TIB molecules on HSA were deleted.

Docking of PNPP with HSA

The docking calculation of each PNPP conformation with HSA was performed using the UNITY 4.1.2 program package (24). A MOLCAD fast Connolly surface was created for the protein atoms surrounding site II of HSA (within 8 Å from MYR-1003 and MYR-1004). We defined a UNITY surface volume constraint expanded by a tolerance of 1.0 Å from the MOLCAD surface. The PNPP molecule should be the inside of the surface volume constraint. Hydrogen bond donors in protein atoms surrounding site II were defined as UNITY queries. However, this was not done for hydrogen bond acceptors because there are no donors in PNPP. The PNPP molecule should form a hydrogen bond to Arg-410, which is important for esterase-like activity (10), and to at least one of the other residues. However, Tyr-411 was not included in the UNITY queries because it is a catalytic residue. A hydrogen bond tolerance was set within 0.5 Å. We searched for conformers that satisfied the above conditions using a UNITY 3D Search and obtained only one hit. In this search, the HSA template and each PNPP conformer were kept rigid. Finally, the docked HSA–PNPP complex was minimized in a same manner as above. AM1-Wang-Ford charges (25,26) were generated as charges for the PNPP using CS MOPAC (27).

RESULTS AND DISCUSSION

Species Differences in Esterase-Like Activity

pH Profiles

The pH profiles for k_{cat}, K_S, and k_{cat}/K_S for the hydrolysis of PNPA by the different types of SA were constructed. The k_{cat} values are markedly dependent on pH in all species examined (data not shown) and show that the susceptibility of the active sites to nucleophilic attack increases with pH. In contrast, the K_S values decrease with increasing pH (data not shown). It has been reported that pH-dependent conformational changes (N–B transition) occur in albumin when going from neutral to slightly alkaline pH (28). Therefore, alterations in nucleophilic attack in active sites and in the affinity to PNPA could be due, totally or partly, to changes in the tertiary structure of albumin, which accompany the pH-dependent N–B transition. The k_{cat}/K_S values increase with raising pH (data not shown). This difference in activity appears to be mainly due to differences in k_{cat}.

The results of the pH experiments indicate that nucleophilicity in the active sites is very important in catalysis activity. The pK_a value for the hydrolysis of PNPA with various albumins is calculated, and the result for human is shown in Fig. 2, as a typical example. It is lowest in human albumin, in which, however, the highest enzymatic activity was found. Horse albumin has the highest pK_a value for hydrolysis but has the lowest enzymatic activity (Table I). These results indicate that hydrolytic activity is dependent on the pK_a and on the nucleophilicity of the most important catalytic residue, Tyr-411, in the various albumins. According to X-ray crystallographic studies of HSA, the oxygen of the hydroxyl group of Tyr-411 is adjacent to the nitrogen of the guanidine group of Arg-410 (10). Therefore, the species differences in esterase-like activity could be affected by structural changes after substrate binding.

On the other hand, Tyr-411 and Arg-410, which are essential for hydrolytic activity, are perfectly preserved in all species examined to date, and the amino acid residues in the immediate vicinity of Tyr-411 residue are highly conserved among species. Thus, the significant decrease in reactivity in rat and horse albumin must reflect differences in the microenvironment in the active sites rather than a deficiency of basic activity. The SA with a lower pK_a can cause an easier

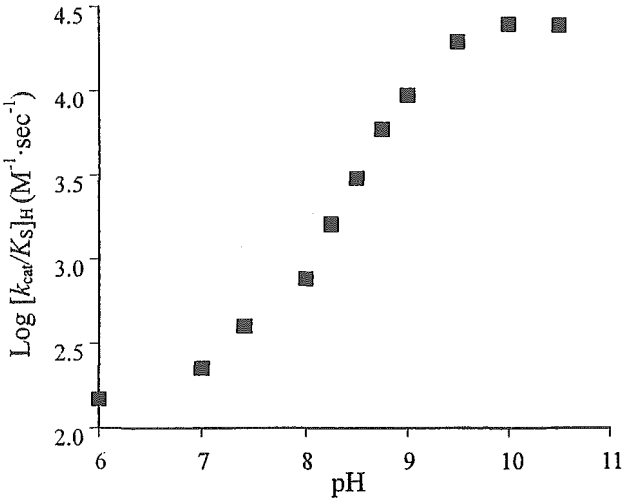

Fig. 2. Plots of $[\log k_{cat}/K_S]_H$ against pH for the hydrolytic reaction of PNPA with HSA. Reaction conditions: 1/15 M phosphate buffer at 25°C. Plots represent mean ± SD (n = 3).

dissociation of Tyr-411 residue, which can be predicted to have a higher reactivity in consequence.

Energy and Thermodynamic Profiles

Using the hydrolysis parameters, we calculated the energy changes for the different albumins at pH 7.4. ΔG_T, considered to be an indicator of the energy difference, is dependent on ΔG and can be calculated from k_{cat} (Table II). As described above, the hydrolysis reaction of albumin is k_{cat} dependent. This observation suggests that the esterase activity is due to the energy difference between the substrate–protein complex in the transition state (ES*) and in the ground state (ES).

The free-energy diagram for the hydrolysis of human and horse albumins toward PNPA is given in Fig. 3. The different esterase-like activity of various albumins is due to differences in ΔG, namely, the extent of stabilization of the transition state.

In order to investigate the characteristic feature of hydrolysis with respect to PNPA in greater detail, thermodynamic studies on this catalytic reaction were performed. As given in Table II, ΔG, mentioned above, which reflects the activity difference, is due to an entropy change (ΔS). Hence, the entropic difference between the ground state and the transition state is especially significant for hydrolytic reactions of albumin. The active site of HSA to which the substrate binds, having a perfect orientation to the binding site of the substrate (the ester portion) for the hydrolysis, shows a smaller difference in entropy between the transition state and the ground state. This is the reason why hydrolysis by HSA proceeds more readily than by the other albumins. Otherwise, the entropic difference can be also explained by the differences in the effects on water structure in active site(s).

Table I. pK_a Values for the Catalytic Group in Various Albumins

Species	pK_a
Human	9.2
Bovine	9.3
Rabbit	9.4
Dog	9.4
Rat	9.5
Horse	9.7

Average of values from 2–3 experiments, which coincided with each other within 2%.

Table II. Free Energy and Other Thermodynamic Parameters for the Hydrolysis of PNPA by Various Albumins*

Species	ΔG_T (kcal/mol)	ΔG_S (kcal/mol)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
Human	13.9	−5.0	18.9	15.8	−3.2
Bovine	14.5	−4.6	19.1	15.3	−3.9
Rabbit	14.6	−4.6	19.2	15.0	−4.2
Dog	14.9	−4.8	19.7	15.3	−4.4
Rat	15.5	−5.1	20.6	14.8	−5.7
Horse	15.7	−5.1	20.8	14.9	−5.9

PNPA, P-nitrophenyl acetate.
* Reaction conditions: 1/15 M phosphate buffer (pH 7.4) at 25°C. Average of values from two experiments, which coincided with each other within 6%.

Structure–Activity Relationships

Significant variations in hydrolytic activity were found for different substrates, even if they contained the same functional group. Structure–activity studies with enzymes have made it relatively easy to define the structure and the physicochemical features of the active sites by measuring the effects of changes in the structure of the substrate on its interaction with the enzyme (14).

In order to characterize the active site(s) of HSA toward PNPA, the reactions of *p*- and *o*-nitrophenyl esters with HSA were investigated kinetically and thermodynamically. The kinetic parameters for the hydrolytic reactions of nitrophenyl esters with HSA are listed in Table III. The values of K_S

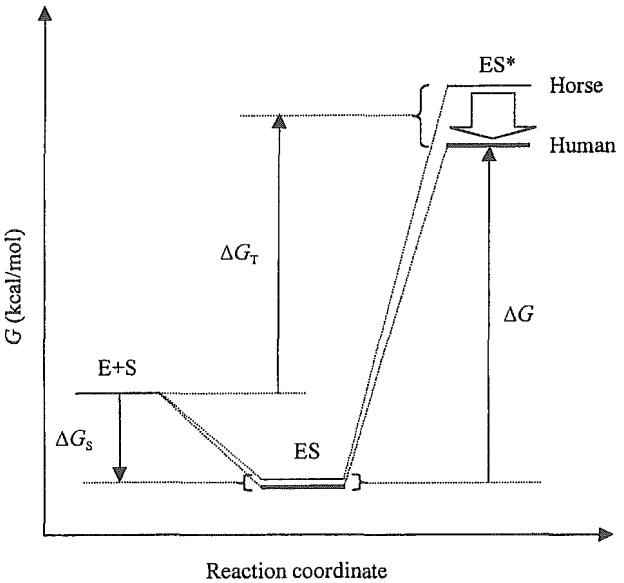

Fig. 3. Comparison of the free-energy profiles of the hydrolytic reaction of PNPA with albumin from human and horse. ΔG_T, free-energy differences; ΔG_S, free-energy change for the initial reaction of albumin and PNPA; ΔG, activation free energy.

Table III. Kinetic Parameters for the Hydrolysis of *p*- and *o*-Nitrophenyl Esters by HSA*

Substrate	Number of C in side chain	k_{cat} (10^{-3} s^{-1})	K_S (10^{-2} mM)	k_{cat}/K_S (M^{-1} s^{-1})
p-nitrophenyl ester				
PNPA	C_2	86.8 ± 4.8	21.7 ± 0.3	403.4 ± 35.6
PNPP	C_3	166.2 ± 12.2	15.5 ± 0.2	1079.3 ± 135.8
Butyrate	C_4	19.3 ± 0.8	7.5 ± 0.1	261.0 ± 23.9
Valerate	C_5	2.8 ± 0.1	4.5 ± 0.1	63.3 ± 6.2
Capronate	C_6	1.2 ± 0.1	2.3 ± 0.1	55.0 ± 19.0
Caprylate	C_8	1.1 ± 0.2	0.9 ± 0.0	133.7 ± 43.6
Caprinate	C_{10}	0.6 ± 0.0	1.0 ± 0.0	61.0 ± 14.4
Laurate	C_{12}	0.3 ± 0.0	1.3 ± 0.0	25.7 ± 6.8
Trimethyl acetate	C_5	0.4 ± 0.2	11.3 ± 0.3	3.1 ± 1.0
o-nitrophenyl ester				
Acetate	C_2	3.3 ± 0.6	10.7 ± 0.0	30.6 ± 4.6

HSA, human serum albumin; PNPA, *p*-nitrophenyl acetate; PNPP, *p*-nitrophenyl propionate.
* Reaction conditions: 1/15 M phosphate buffer (pH 7.4) at 25°C. The concentration of substrate is 5 mM and that of HSA is 25–150 mM. Each value is shown as mean ± SD. (n = 4–5).

decreased with increasing length of the side chain. Up to *p*-nitrophenyl valerate (a five-carbon side chain) a linear decrease in K_S values against the increase in the number of carbons in the side chain was found, showing that the hydrophobic side chain of the substrate influences the hydrolytic reaction. However, the decrease in k_{cat}/K_S and k_{cat} values, depending on the incremental length of the side chain, was not a decrease but a pronounced increment in the case of PNPP (a three-carbon side chain). This effect was one of affinity to albumin. Corresponding to the hydrolytic differences of the various albumins, the k_{cat}-dependent hydrolytic reaction catalyzed by albumin is due to the directing properties of binding to the substrate rather than affinity to albumin. It is conceivable that PNPP has a structure that is more suitable to the active expression of albumin, compared with other *p*-nitrophenyl esters. However, among structural isomers with the same molecular weight, such as PNPA and *o*-nitrophenyl acetate or *p*-nitrophenyl valerate and *p*-nitrophenyl trimethylacetate, substantial differences in activities were observed, which are believed to be related to steric exclusion caused by the different branches and sites of branching in the side chain.

The findings relative to the reactions of *p*-nitrophenyl esters with HSA are consistent with the above experimental studies on species differences using PNPA, suggesting that hydrolytic activity is dependent on the activation free energy (ΔG) (Table IV). Differences in the free-energy diagrams for the hydrolysis of PNPP vis-à-vis capronate can be illustrated as following the same pathway as shown in Fig. 3, where the transition state of capronate–HSA intermediate (ES*) has the highest energy of all the *p*-nitrophenyl esters. Stabilization of the transition state is an important aspect of the hydrolytic reaction, and ΔG is an indicator of the esterase-like activity of albumin. As a marker of chemical conversion, k_{cat} is more significant for hydrolytic activity than K_S, which can be thought of as an index of affinity for the substrate.

The magnitude of the activation free energy (ΔG) is related to the magnitude of the activation entropy change (ΔS) as seen from the thermodynamic analysis (Table IV). Hence, because of the entropic difference between the enzyme–substrate complex states (ES) and the transition state (ES*), PNPP has the minimum value of the related nitrophenyl ester derivatives tested and, therefore, has the highest activity in this study. That is, PNPP has an optimal structure and a more perfect orientation for binding to albumin than the other esters during the hydrolytic reaction.

Considering the findings of hydrolytic differences between various species, it can be concluded that a ΔS dependence of the transition state stabilization is significant for albumin hydrolysis and that the binding of the substrate to albumin is a contributing factor that determines activity.

Structural Mechanism of Esterase-Like Activity

The catalytic reaction of serine-proteases, a primary hydrolase, can be divided into two processes. Initially, the enzyme and substrate associate to form a noncovalent enzyme–

Table IV. Free Energy and Other Thermodynamic Parameters for the Hydrolysis of Esters by HSA*

Substrate	Number of C in side chain	ΔG_T (kcal/mol)	ΔG_S (kcal/mol)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
PNPA	C_2	13.9	−5.0	18.9	15.8	−3.1
PNPP	C_3	13.3	−5.2	18.5	16.3	−2.2
Butyrate	C_4	14.2	−5.6	19.8	14.3	−5.5
Valerate	C_5	15.0	−5.9	21.0	14.7	−6.2
Capronate	C_6	15.1	−6.3	21.5	12.8	−8.7

HSA, human serum albumin; PNPA, *p*-nitrophenyl acetate; PNPP, *p*-nitrophenyl propionate.
* Reaction conditions: 1/15 M phosphate buffer (pH 7.4) at 25°C. Average of values from two experiments, which coincided with each other within 4%.

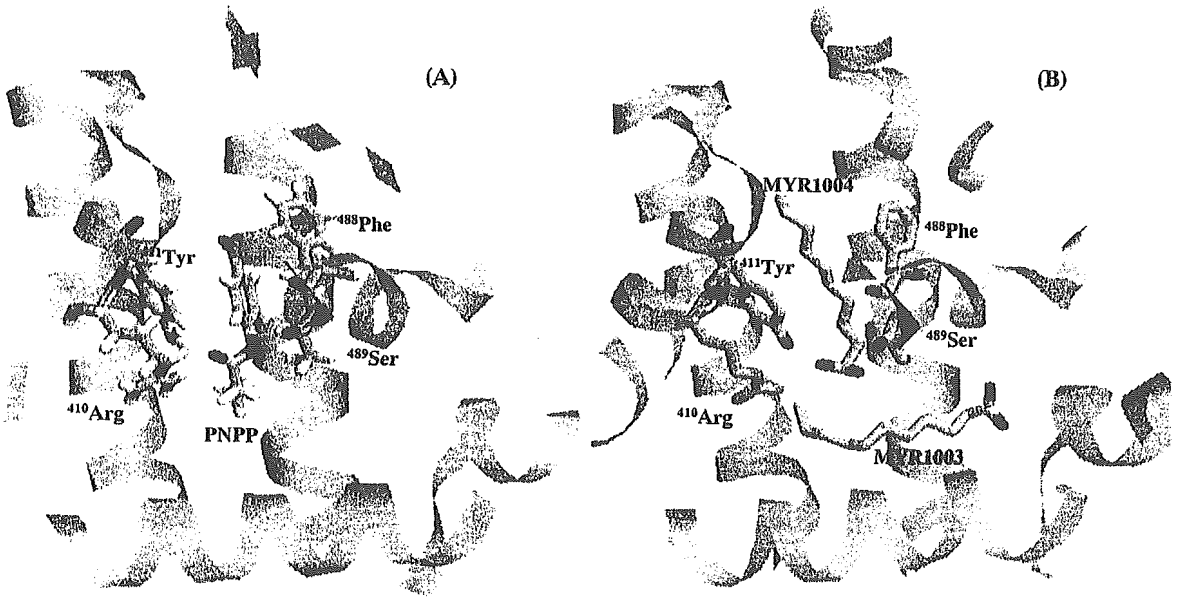

Fig. 4. Binding model for PNPP in site II of HSA (A) and the X-ray crystallographic structure of the HSA–myristate (MYR) complex in site II of HSA (B). The illustration was made with the RasMol program.

substrate complex, which is held together by interactions between the hydroxyl group of the serine residue and the substrate (referred to as a catalytic triad). This complex represents the first tetrahedral intermediate. Water is then involved in the formation of the enzyme–product complex via a second tetrahedral intermediate with the acyl-serine. This is followed by transacylation from serine to water. In addition to this catalytic triad mechanism, a region of the enzyme, termed the oxyanion hole, has been reported to exist and which promotes catalysis by providing transition-state stabilization (29).

The catalytic reaction between HSA and PNPA can be expressed quantitatively using the Michaelis–Menten equation. Means et al. reported that, after combining rapidly and reversibly into an active albumin–substrate intermediate, *p*-nitrophenol is generated and rapid acetylation occurs at the reactive residue, Tyr-411 (2). Ohta et al. observed that the second reaction, deacetylation of acetyl-albumin, is considerably slower than the first step (13). Except for these findings, little is known concerning the esterase-like activity of HSA. For example, there have been no reports concerning the possible presence of a catalytic triad or an oxyanion hole, as has been reported for serine-proteases.

In order to elucidate the detailed catalytic mechanism of the esterase-like activity of HSA, the influence of deuterium oxide on the reactivity was investigated. Subsequently, a complex of PNPP, showing the highest activity among the *p*-nitrophenyl esters tested, and HSA was constructed using computer modeling techniques.

Isotope Effect of Deuterium Oxide

The involvement of a water molecule and proton transfer can be evaluated by measurement of the deuterium oxide isotope effect (14). The influence of this molecule on the hydrolysis of PNPA by HSA was investigated to track the behavior of the proton (data not shown). The result can be regarded as a reflection of the acetylation reaction that occurs during the first step of the hydrolytic reaction, generating *p*-nitrophenol. Because no significant deuterium oxide isotope effect could be detected, this implies that a water molecule is not involved in the hydrolysis catalyzed by HSA. More specifically, the first step of the hydrolysis does not proceed via general acid or base catalysis, but is predominantly due to the nucleophilic attack of Tyr-411 residue.

The same proton exchange technique has been used to investigate the catalytic mechanism of many serine proteases (30). In these cases, the reactivity was dependent on the mole fraction of deuterium present, suggesting that the isotope effect observed for these reactions originated from the active-site serine hydroxyl group (i.e., the reactions involved the formation of catalytic triads). The current results are in contrast to these findings and imply that a catalytic triad is not involved in the hydrolysis of PNPA by HSA.

On the other hand, using *N*-trans-cinnamoylimidazoles containing amide bonds as model substrates, during the second step of hydrolysis, the deacylation rate of the cinnamoyl–albumin was found to be about 3- to 4-fold smaller in deuterium oxide than in water (13). This deuterium effect indicates that a water molecule plays a role in the deacylation reaction. Therefore, general acid or base catalysis rather than nucleophilic catalysis predominates in the deacylation of cinnamoyl–albumin. The deacetylation of acetyl-albumin, in the second step of the hydrolysis of PNPA, appears to proceed according to the same mechanism as *N*-trans-cinnamoylimidazoles.

In summary, the hydrolysis of PNPA by HSA could take place as follows: HSA is initially acetylated as the result of a nucleophilic attack of Tyr-411 on the substrate, and the deacetylation of acetyl-albumin then proceeds via general acid or base catalysis involving a water molecule.

Three-Dimensional Structure of the Modeled HSA–PNPP Complex

In the model of HSA–PNPP complex, the binding of PNPP was similar to that of MYR-1004 in the X-ray structure

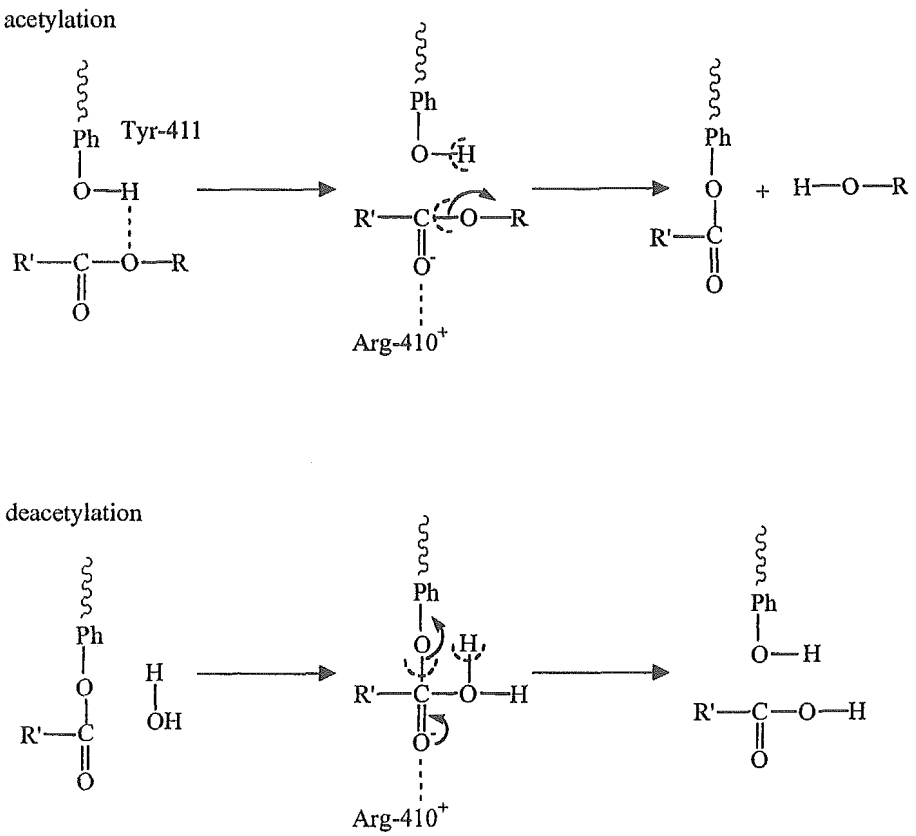

Fig. 5. Simulated mechanism for the hydrolysis of PNPP by HSA.

(16) (Fig. 4). The *p*-nitrophenyl moiety of PNPP is bound to the hydrophobic pocket of MYR-1004 in the X-ray structure. In addition, the *p*-nitrophenyl moiety is associated with the aromatic residues of Tyr-411 and Phe-488. The hydrogen bond between the hydroxyl group of Ser-489 and the carboxyl oxygen atom of PNPP stabilizes the complex. The ethyl moiety of PNPP is bound to a part of the hydrophobic pocket for MYR-1003 in the X-ray structure. The distance between the phenoxyl oxygen atom of Tyr-411 and the carbonyl carbon atom of PNPP was found to be about 3.8 Å and might enable a nucleophilic reaction. The side chain of Arg-410 forms a hydrogen bond with the carbonyl oxygen atom of PNPP, and this hydrogen bond could function as an oxyanion hole. Moreover, The distance between the phenoxyl oxygen atom of Tyr-411 and the carbonyl oxygen atom of PNPP was about 2.8 Å, thus favoring a hydrogen bond formation. This hydrogen bond could account for isotope effect observed for the acetylation of HSA (Fig. 5). This model is consistent with the experimental results; that is, Tyr-411 as the catalytic residue (2,10) and the importance of Arg-410 (10), as mentioned above.

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