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論文内容の要旨

Soluble guanylate cyclase (sGC), catalyzing the conversion of GTP to cGMP, is a heme-containing enzyme distributed in animal brain and lung. It has been pointed out that sGC is one of the receptor proteins for nitric oxide (NO), which acts as a signal molecule to play various physiological roles in smooth muscles, platelets, brain and macrophages. Indeed, it has been reported that partially purified sGC was activated about 70 fold in terms of enzymatic activity by addition of NO or NO releasing agents. The activation was attributed to ligation of NO to a heme group of sGC, but spectroscopic studies of sGC have hardly been done, because a content of sGC in animal tissues is much lower than that of other well-known hemoproteins. To obtain useful data for structural analysis from resonance Raman spectroscopy, 70 μl of 30 μM solution is required for a single measurement and thus more than 1 mg of sGC is required. To get a sufficient amount of purified enzymes, a method for large scale purification has to be established first.

A reported method about purification of sGC involves several kinds of column chromatography. To purify sGC from bovine lung, they tried the reported method, but the yield was so low that they could not observe absorption spectra of the purified sGC as well as resonance Raman spectra. Therefore, they screened chromatography media for each step of purification. This process included a time-consuming trial and error method but finally they succeeded in purifying a large amount of sGC by the use of affinity column prepared by themselves. The SDS-PAGE analysis of the purified sGC indicated that the enzyme is two distinct subunits having molecular weight of 69,000 and 74,000. The absorption band of sGC as purified appeared at 431 nm but it was shifted to 399 nm by the addition of NO. Activity of the purified sGC was increased up to 150-fold in the presence of NO, compared to that in its absence, while heme-deleted sGC did not show such an activation upon the addition of NO. These results mean that the sGC thus purified in our laboratory is essentially the same as the enzyme purified using the immunoaffinity column. The heme content of the present sGC was determined to be 0.8 heme per heterodimer. The heme is presumably bound to His-105 of β subunit judging from mutation studies of recombinant sGC.

Using the purified sGC, they measured resonance Raman spectra of the sGC. Resonance Raman spectroscopy is a very effective tool to elucidate a heme structure of hemoproteins. What is especially superior to other spectroscopic methods is that an Fe-Ligand vibration can be observed in raw spectra. Their results meant the sGC as isolated contained the histidine-coordinated ferrous high spin heme but when NO gas was incorporated to the sGC, the Fe-His bond was cleaved to form the five-coordinated heme-NO complex. The low Fe-His stretching frequency observed at 203 cm^{-1}

indicated the absence of hydrogen bonding proximal His and weak Fe-His bond. Since the Raman spectrum of sGC-NO was distinct from those of model compounds in spite of the absence of a covalent bond between the heme-NO complex and the protein matrix, the five-coordinated heme-NO complex must be bound to the sGC protein tightly. Furthermore, the Fe-CO stretching mode was observed at 473 cm^{-1}, which are extremely lower than those of other hemoproteins, and the Fe-NO and N-O stretching modes of sGC-NO were observed at 521 and 1681 cm^{-1}, respectively. The assignments are based on the frequency shift upon NO-isotope substitution.

Although the Fe-His bond cleavage has attracted attention with regard to a regulation mechanism of sGC's activity, they assumed that the heme-NO complex in the protein itself has an important role to regulate the sGC activity, because addition of heme-NO complex to its apo protein brought about the same results as the case of the addition of NO to the holoenzyme. Accordingly, they focused on attention to find some changes in Raman spectra of sGC upon additions of a substrate, its analogues and products. In consequence, it was observed for the first time that Raman spectrum of sGC-NO was changed in the presence of cGMP, a product of the enzymatic reaction. The effect of cGMP was seen only for the NO adduct of sGC but not for the CO-bound and unligated forms. The Fe-His stretching mode of the resting sGC remained unchanged upon addition of cGMP. It was noted that additional N-O stretching mode appeared but Fe-NO stretching mode remained unaltered in the presence of cGMP. Interestingly, these results imply the existence of some interactions between NO and distal side residues in the heme pocket, which is provoked by the association of cGMP to sGC. In addition, the additional N-O stretching band, which is of higher frequency than that of other enabled them to predict fast dissociation of NO from heme on the basis of model compound studies. In general, five-coordinated heme-NO complexes are more stable than six-coordinated heme-NO complexes due to *trans* effects. However, NO dissociation rate of sGC-NO was larger than that of myoglobin-NO by a factor of two. Therefore, the distal side of sGC heme pocket was assumed to have an anionic character, yielding some repulsive interactions to NO bound to the heme. When cGMP was injected into sGC-NO, NO dissociation was accelerated by 10 times and the fast and slow phases ratio was dependent on the concentration of cGMP. They interpreted it as a kind of feedback inhibition. This cGMP effect is not so high and therefore may not work as a main regulation circuit, because NO dissociation is not so fast that could adjust the enzymatic activity. Some change of the Fe-His bond similar to the case of the R-T transition of hemoglobin is required to cause rapid dissociation of NO. Although an endogenous inhibitor has never been identified yet, it is widely believed that an endogenous inhibitor may have a key role for down regulation of sGC through the proximal His.

For more comprehensive understanding the structure-function relationship of heme of sGC, it is necessary to carry out a number of experiments with suitable model compounds. They selected myoglobin, one of the most simple hemoproteins, as a relevant model for understanding the meaning of frequencies of ligand vibrations. Although Mb-NO is of six-coordinate at neutral pH, showing the Fe-NO and N-O stretching modes at 556 and 1613 cm^{-1}, respectively, it is found to change into a five-coordinated complex like sGC at pH 4, showing Fe-NO and N-O stretching modes at 520 and 1668 cm^{-1}, respectively. Subsequently they prepared several kinds of recombinant Mb mutants expressed by *E. coli* and measured Raman spectra of its NO adduct to determine Fe-NO and N-O stretching modes. Wild type sperm whale Mb showed the Fe-NO and N-O stretching modes at 560 and 1613 cm^{-1}, respectively. Except for subtle differences in the Fe-NO stretching frequency, there were no differences between sperm whale Mb and horse heart Mb. Accordingly, they decided to use wild type sperm whale Mb as a standard protein. Substitution of the distal His (His64) to an aprotic amino acid such as Gly or Leu caused the higher frequency shift of the N-O stretching mode by 20-26 cm^{-1} but negligible shift for the Fe-NO stretching mode. This must be a charge effect of distal residues. In contract, point mutation of Val68 or Leu29, which are distal side amino acid residues close to distal His, to a bulky amino acid, Trp, caused the frequency shift of the Fe-No stretching mode by 5-10 cm^{-1} but no change for the N-O stretching mode. This shift was attributed to distortion of the Fe-N-O geometry due to steric hindrance. These data obtained with Mb-NO will be ruminated to reveal the structural implication of the Raman data of sGC-NO.

論文の審査結果の要旨

本論文は可溶性グアニル酸シクラーゼを精製し、共鳴ラマンスペクトルを測定して、そのデータの解釈のためミオグロビンの部位特異的アミノ酸置換体のNO錯体を調べたことが主たる内容で、それ以外にチトクロムP450のイソシアニド錯体について調べた結果も含まれる。本内容に関して既に２報が印刷され、１報が投稿中である。

論文は英文で書かれ５章からなる。第１章ではグアニル酸シクラーゼの生理学的意味、それが膜結合型と可溶性と２種あって臓器により分布が違うこと、そして可溶性のもののみが一酸化窒素(NO)で活性化を受けること、等本研究の背景となる生化学的研究成果をまとめてある。またヘム-NO錯体のこれまでの知識もまとめられていて、研究の位置づけは十分できていることが分かる。第２章と第３章が可溶性グアニル酸シクラーゼ(sGC)に関する記述で、第２章は酵素の精製の仕方及びその生化学的キャラクタリゼーション、第３章はその共鳴ラマンスペクトルの測定結果とそれに基づく本酵素の活性制御機構に関する新しい提案を含む。

本酵素の精製法はまだ確立しておらず、１回の実験で１mg以上のタンパク標品を得る方法として報告されているやり方を実施してもうまくいかないので、申請者が新しい方法を確立した。その本質はGTPセファローズカラムを自分で調整し、それをイオン交換クロマトグラフィーの次に入れたこと、最後にハイドロキシアパタイトのカラムを通すことの２点である。この新しい方法で精製したsGCの活性を、放射線ラベルGTPを用い生成物のcGMPを定量することにより追跡した。その結果、ウシ肺の破砕物溶液に比べて12500倍の比活性を持つ酵素を得た。精製標品はα、βダイマー構造で、ヘム定量の結果はダイマー当たり１個のヘムを持つこと、ダイマー当たり１つの触媒部位を持っていて、NOを入れると活性が150倍上昇した。この酵素が一般のGTP結合タンパクと同様にADPリボシル化を受けることを初めて見つけた。

第３章では精製標品がヒスチジンの配位した５配位ヘムを持つが、そのFe-His結合はヘムタンパクとしてはかなり弱いこと、NOが配位するとFe-His結合が切れ、NOの配位した５配位ヘムができるという共鳴ラマン分光らしい結果を得た。N-O伸縮振動やFe-NO伸縮振動を同位体シフトで同定し、基質存在下でNO伸縮振動がシフトすることを初めて見つけ、それが生成物であるcGMPの結合によることを突き止めた点は高く評価される。そしてcGMP存在下ではNOの解離速度が１０倍速くなることから、生成物によるフィードバック阻害という本酵素の制御機構を提案している点はオリジナルで意義高い。

cGMPの結合によりN-O伸縮振動が高波数シフトするがFe-NO伸縮振動がシフトしない事実を説明するため、第４章ではミオグロビンのアミノ酸置換体のNO錯体の共鳴ラマンスペクトルを調べた。sGCのヘムはタンパクに強く固定され、cGMPの結合によりタンパクとNOとの水素結合が切れるといったことが起こっていて、立体障害は起こってないと結論した。第５章はチトクロムP450イソシアニド錯体の共鳴ラマンの研究成果が述べられている。ここではイソシアニドの^{15}N同位体を自分で化学的に合成し、チトクロムP450のFe-イソシアニド伸縮振動を初めて検出した。カンファーP450とNO還元P450に適用し、その２種が違う振動数を持つことを指摘した。

以上、本論文の内容は国際的に最前線の非常におもしろい研究成果であり、学位論文と

して十分であるということで全員の意見が一致した。

また申請者に研究成果を約１時間で説明させ、それに対して２時間の質疑応答による口述試験を行った。申請者は第１～第４章の内容を分かりやすく説明した。生理的な側面からの質問、酵素化学的な質問および物理化学的な質問に関しても、それぞれ的確な返答を行った。学力に関しては、学位論文に記載された内容ならびに関連分野の知識を十分に備えていることが明らかになった。論文は分かりやすい英語で書かれており、また結果の一部は外国の雑誌に印刷されているので、英語力も十分であると判断した。公開発表会においても成果を分かりやすく説明し、個々の質問に対しても的確に答えたので、審査委員会は全員一致で試験に合格と判断した。