The β and γ subunits play distinct functional roles in the α₂βγ heterotetramer of human NAD-dependent isocitrate dehydrogenase

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Human NAD-dependent isocitrate dehydrogenase existing as the α₂βγ heterotetramer, catalyzes the decarboxylation of isocitrate into α-ketoglutarate in the Krebs cycle, and is allosterically regulated by citrate, ADP and ATP. To explore the functional roles of the regulatory β and γ subunits, we systematically characterized the enzymatic properties of the holoenzyme and the composing αβ and αγ heterodimers in the absence and presence of regulators. The biochemical and mutagenesis data show that αβ and αγ alone have considerable basal activity but the full activity of α₂βγ requires the assembly and cooperative function of both heterodimers. α₂βγ and αγ can be activated by citrate or/and ADP, whereas αβ cannot. The binding of citrate or/and ADP decreases the S₀.₅, isocitrate and thus enhances the catalytic efficiencies of the enzymes, and the two activators can act independently or synergistically. Moreover, ATP can activate α₂βγ and αγ at low concentration and inhibit the enzymes at high concentration, but has only inhibitory effect on αβ. Furthermore, the allosteric activation of α₂βγ is through the γ subunit not the β subunit. These results demonstrate that the γ subunit plays regulatory role to activate the holoenzyme, and the β subunit the structural role to facilitate the assembly of the holoenzyme.

Mitochondrial NAD-dependent isocitrate dehydrogenases (NAD-IDHs, EC 1.1.1.41) in eukaryotes are a family of enzymes that catalyze the oxidative decarboxylation of isocitrate (ICT) into α-ketoglutarate (α-KG) while reducing NAD to NADH in the rate-limiting step of the Krebs cycle. Those enzymes are conserved from yeast to mammals and consist of multiple subunits. They are all allosteric enzymes under strict cellular regulation, which can be activated by AMP (in yeast) or ADP (in mammals) and citrate (CIT) but inhibited by ATP (in mammals) and NADH. Thus, the cellular ratios of [ATP]/[AMP or ADP] and [NADH]/[NAD] can regulate the activity of NAD-IDH in the Krebs cycle and influence the energy production and substance flux in cells.

Mammalian NAD-IDHs isolated from porcine heart, porcine liver, bovine heart, and ox brain have been biochemically characterized. All these enzymes are consisted of three types of subunits in the ratio of 2α:1β:1γ, and exist as the α₂βγ heterotetramer composed of the αβ and αγ heterodimers, which can be further dimerized into a heterooctamer (the heterotetramer and heterooctamer are sometimes called holoenzyme and hereafter we will not distinguish the heterotetramer, heterooctamer and holoenzyme in the biochemical context unless otherwise specified). The α, β and γ subunits have molecular masses of 37 kDa, 39 kDa and 39 kDa, respectively, and exhibit distinct isoelectric points. The α and β subunits share about 40% sequence identity, the α and

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γ subunits about 42% sequence identity, and the β and γ subunits about 52% sequence identity. The previous biochemical studies showed that the individual α, β or γ subunit of porcine heart NAD-IDH isolated using urea (2M) is either inactive or exhibits very low activity, but the recombined α and β subunits or α and γ subunits in the form of the αβ or αγ heterodimer shows considerable activity. All mammalian NAD-IDHs require divalent metal ions such as Mn2+, Mg2+, Co2+, and Cd2+ for the activity. In addition, the activity could be positively regulated by CIT and ADP but inhibited by ATP and NADPH. It was suggested that these enzymes have two binding sites per heterotetramer for each ligand, including CIT, Mn2+, NAD, and ADP.

Recombinant human NAD-IDH was successfully expressed in E. coli, which allowed more mutagenesis and biochemical studies of the enzyme based on sequence alignment with E. coli and porcine mitochondrial NADP-IDHs. Those studies examined the functional roles of several strictly conserved residues of the α, β and γ subunits in the bindings of metal ion, ICT, NAD, and ADP, and showed that the α subunit is critical for the catalytic activity, and the β and γ subunits play functional roles in allosteric regulation; however, the α subunit alone has no detectable activity and its coexistence with one of the two regulatory subunits is essential for the activity.

Saccharomyces cerevisiae NAD-IDH exists as a heterotetramer composed of two heterodimers of the regulatory subunit IDH1 and catalytic subunit IDH2, which can be further assembled into a heterooctamer. The structures of yeast NAD-IDH in apo form, in CIT bound form, and in CIT-AMP bound form have been determined at moderate resolution, which revealed the assembly of the heterotetramer and heterooctamer and the binding sites of the activators. IDH1 and IDH2 form a compact heterodimer that acts as the basic functional and structural unit, and the structural communication between the two subunits is considered to be important to the allosteric regulation.

As human NAD-IDH contains three types of subunits, the molecular mechanism of allosteric regulation appears to be more complex than that of yeast NAD-IDH. In addition, the enzymatic properties of the composing αβ and αγ heterodimers and the exact functional roles of the β and γ subunits in the αβγ heterotetramer are not well understood. Thus, human NAD-IDH is a very good model for studying the molecular mechanism of allosteric regulation. Moreover, mutations of human NADP-dependent IDHs localized in the cytoplasm and mitochondria (also called IDH1 and IDH2) have been identified in multiple types of tumors and the mutant proteins confer neomorphic activity to convert α-KG into 2-hydroxylglutarate (2-HG) whose accumulation can result in epigenetic dysregulation, leading to oncogenesis and development of tumors. Intriguingly, human NAD-HAD-IDH (also called IDH3) has also recently been implicated in some diseases. Homozygous mutations of the β subunit are suggested to be a cause of retinitis pigmentosa, a hereditary degeneration of the retina leading to blindness. Aberrant expression of the α subunit can promote malignant tumor growth by inducing HIF-1-mediated metabolic reprogramming and angiogenesis. Abnormalities of human NAD-IDH are associated with pathogenesis of major psychiatric disorders. Furthermore, human NAD-IDH is shown to be a novel target of tributyltin, an environmental contaminant chemical, in human embryonic carcinoma cells. Thus, the structural and mechanistic studies of human NAD-IDH have also important biomedical implications.

In this work, we systematically studied the enzymatic properties the αβ and αγ heterodimers and the αβγ heterotetramer of human NAD-IDH in the absence and presence of several regulators including CIT, ADP, and ATP. Our biochemical data show that the αγ heterodimer has similar enzymatic properties and kinetic parameters as the αβγ heterotetramer, whereas the αβ heterodimer has only basal activity and cannot be allosterically regulated. The catalytic efficiencies of the αβγ and αγ enzymes are activated by CIT or ADP through decreasing the S0.5 for ICT. Additionally, the two activators can function synergistically, suggesting that CIT and ADP can bind to the enzymes independently and cooperatively. Moreover, ATP can activate the αβγ and αγ enzymes at low concentration in a manner similar to ADP and inhibit the activities at high concentration, but has only inhibitory effect on the αβ enzyme. Furthermore, the mutagenesis data show that the αβ and αγ heterodimers contribute equally to the activity of the αβγ heterotetramer in either the absence or presence of the activators, and the allosteric regulation of the αβγ heterotetramer is through the γ subunit not the β subunit. These results together demonstrate that the γ subunit plays the regulatory role to activate the holoenzyme, and the β subunit the structural role to facilitate the assembly of the holoenzyme. These findings provide new insights into the molecular mechanisms of the function and allosteric regulation of mammalian NAD-IDHs.

Results
Preparation of human NAD-IDH. The αβ and αγ heterodimers and the αβγ heterotetramer of human NAD-IDH were prepared as described in “Methods”. In vitro assembly of the αβγ heterotetramer from the αβ and αγ heterodimers gave a much higher yield than the co-expression method, and the αβγ heterotetramer obtained using both methods exhibited no difference in enzymatic properties, and thus we used the assembled αβγ heterotetramer in all the biochemical studies. SDS-PAGE analyses showed that the purified αβγ protein and αβ and αγ proteins are of sufficient purity (>95%) and there are two protein bands with equal intensity that are characteristic of mammalian NAD-IDHs, with the upper band corresponding to the β or/and γ subunits (39 kDa) and the lower band corresponding to the α subunit (37 kDa) (Fig. 1a). Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) analyses showed that the αβγ protein exhibits an elution volume of about 11 ml with an average molecular mass of about 288 kDa corresponding to a dimer of heterotetramer or a heterooctamer (theoretical molecular mass of 304 kDa), which is in agreement with the previous reports for the recombinant human NAD-IDH4,24,25 and the isolated pig heart NAD-IDH1. Both the αβ and αγ proteins exhibit an elution volume of about 14 ml at the injection protein concentration of 2 mg/ml with an average molecular mass of about 79 kDa corresponding to the αβ and αγ heterodimers (theoretical molecular mass of 76 kDa), but exhibit an elution volume of about 13 ml at the injection protein concentration of 12 mg/ml with an average molecular mass of about 130 kDa corresponding to dimers of heterodimers (theoretical molecular mass of 152 kDa) (Fig. 1b). These results indicate that the αβγ protein exists as a heteroctamer in solution; the αβ
and αγ proteins exist as heterodimers at low concentration but as dimers of heterodimers at high concentration. As the final protein concentrations in the enzymatic studies were below 0.2 μg/ml, presumably the αβ and αγ proteins exist mainly as heterodimers and the α2βγ protein as a heterooctamer.

Enzymatic properties of human NAD-IDH. The αβγ protein has a specific activity of 20.2 ± 0.3 μmol/min/mg at the standard conditions, and the αβ and αγ proteins exhibit specific activities of 3.33 ± 0.13 μmol/min/mg and 7.27 ± 0.31 μmol/min/mg (about 16.5% and 36.0% of that of the αβγ protein), respectively, which are comparable to those of the isolated pig heart NAD-IDH2 and the purified αβγ enzyme of human NAD-IDH19, but are significantly higher than those of the unpurified human NAD-IDH 22 (Table 1). These results indicate that both the αβ and αγ heterodimers have considerable basal activities with the αγ heterodimer having about 2-fold higher activity than the αβ heterodimer, and the full activity of the αβγ heterotetramer requires the assembly and cooperative functions of both heterodimers.
The previous biochemical data showed that mammalian NAD-IDHs require divalent metal ions for their activities⁹-¹⁹. Consistently, our biochemical data show that the αβγ, αβ and αγ enzymes all require divalent metal ions for their activities but have different Š0.5 values. The αβγ enzyme has a Š0.5 value of 60.2 ± 6.0 μM for Mn²⁺, the αγ enzyme has a slightly higher Š0.5 (1.6-fold), but the αβ enzyme has a significantly higher Š0.5 (88-fold) (Table 2). We also analyzed the effects of different metal ions on the activities of these enzymes, and among the six metal ions tested (Mn²⁺, Mg²⁺, Co²⁺, Zn²⁺, Ni²⁺ and Ca²⁺, Mn²⁺ is the most effective one (Supplementary Table S1). Thus, we used Mn²⁺ in all the activity assays and kinetic studies.

Activation of human NAD-IDH by CIT or/and ADP. The previous biochemical data showed that the activities of mammalian NAD-IDHs can be positively regulated by CIT and ADP¹¹,²⁰. To investigate the activation effects of CIT or/and ADP on the αβγ, αβ and αγ enzymes, we first measured their kinetic parameters in the absence of any regulators. Our results show that the αβγ enzyme has a Vmax of 20.0 ± 0.1 μmol/min/mg, and ŠS0.5ICT, ŠS0.5NAD and ŠS0.5NAD of 2.35 ± 0.05 mM, 60.2 ± 6.0 μM, and 143 ± 5 μM, respectively (Table 2). The αγ enzyme has a Vmax of 7.29 ± 0.11 μmol/min/mg and ŠS0.5ICT, ŠS0.5Me and ŠS0.5NAD of 4.49 ± 0.15 mM, 95.1 ± 3.2 μM, and 238 ± 18 μM, respectively, which are comparable to those of the αβγ enzyme. As a result, the basal catalytic efficiency (kcat/S0.5ICT) of the αγ enzyme is about 19.0% of that of the αβγ enzyme. As the αβ enzyme has a very high ŠS0.5Me and its kinetic parameters could not be obtained at the standard conditions, we measured its kinetic parameters at a substantially increased concentration of MnCl₂ (50 mM). At this condition, the αβ enzyme has a slightly lower Vmax (10.9 ± 0.3 μmol/min/mg) but moderately to substantially higher ŠS0.5ICT (13.4 ± 0.1 mM, 5.7-fold), ŠS0.5Me (3505 ± 314 μM, 88-fold) and ŠS0.5NAD (326 ± 15 μM, 2.3-fold) than the αβγ enzyme, and the basal catalytic efficiency is about 9.5% of that of the αβγ enzyme (Table 2). These data indicate that in the absence of any regulators, the αγ enzyme exhibits similar kinetic properties and has comparable kinetic parameters as the αβγ enzyme, but the αβ enzyme does not.

We then measured the kinetic parameters of the αβγ, αβ and αγ enzymes in the presence of activator(s) (Fig. 2 and Table 3). Compared with those in the absence of any regulators, in the presence of CIT, ADP and both activators, the Vmax of the αβγ enzyme has no significant change but the ŠS0.5ICT is decreased by 1.9, 2.7 and 14.6 folds, respectively; and similarly, the Vmax of the αγ enzyme is slightly increased by 1.2–1.8 folds but the ŠS0.5ICT is decreased by 1.7, 2.7 and 24.7 folds, respectively. In contrast, addition of CIT or/and ADP has no significant effects on the Vmax and ŠS0.5ICT of the αβ enzyme. As a result, the catalytic efficiencies of the αβγ and αγ enzymes are substantially increased by about 15.3 and 44.8 folds, respectively; whereas that of the αβ enzyme is not affected. Thus, the catalytic efficiency of the αγ enzyme is elevated to about 55.6% of that of the αβγ enzyme, while that of the αβ enzyme is only about 0.7% of that of the αβγ enzyme (Table 3). These data indicate that the binding of CIT or/and ADP to the αβγ and αγ enzymes potentiates their activities mainly through decreasing the ŠS0.5ICT without significantly affecting the Vmax and the kcat. As either CIT or ADP can slightly decrease the ŠS0.5ICT of the αβγ and αγ enzymes but the two activators together can substantially decrease the ŠS0.5ICT (Table 3), these data suggest that the two activators can bind either independently or simultaneously to distinct sites at the allosteric site, and the two activators function in a synergistic manner. These results are consistent with the previous biochemical data showing that ADP can positively regulate the activity of mammalian NAD-IDH²¹, and the binding of ADP and CIT can lower the ŠS0.5ICT but has no effect on the Vmax.²⁰,²⁸ These data further demonstrate that in the presence of CIT or/and ADP, the αγ enzyme also exhibits similar kinetic properties as the αβγ enzyme, but the αβ enzyme does not.

Moreover, our kinetic data show that in the absence of any regulators, both the αβγ and αγ enzymes exhibit a Hill coefficient of 2 with respect to ICT, whereas the αβ enzyme exhibits a Hill coefficient of 1, indicating that the
α₂βγ and αγ enzymes contain at least two ICT-binding sites with positive cooperativity whereas the αβ enzyme contains only one ICT-binding site (Table 2). In the presence of CIT, ADP and both activators, the α₂βγ enzyme still exhibits a Hill coefficient of 1.5, 2.0, and 1.5, respectively, indicating a cooperative binding for ICT in all these conditions; whereas the αγ enzyme exhibits a Hill coefficient of 1.2, 1.6, and 1.0, respectively, indicating that there is a cooperative binding for ICT only in the absence of CIT (Table 3). As expected, the Hill coefficient of the αβ enzyme is not affected by CIT or/and ADP. These results suggest that the αγ heterodimer may contain an ICT-binding site each in the α and γ subunits; CIT appears to bind to one site most likely in the γ subunit and thus eliminates the positive cooperativity, but the ADP binding does not occlude the binding of ICT to this site and thus retains the cooperativity. The αβ enzyme contains only one ICT-binding site very likely in the α subunit but no ICT-binding site in the β subunit. As the α₂βγ enzyme exists as a dimer of heterotetramer, there is

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**Figure 2.** Saturation curves of the α₂βγ heterotetramer and the αβ and αγ heterodimers for isocitrate in the absence and presence of positive regulator(s). (a) Saturation curves of the α₂βγ enzyme. (b) Saturation curves of the αβ enzyme. (c) Saturation curves of the αγ enzyme. The activities of the α₂βγ and αγ enzymes were measured at the standard conditions (33 mM Tris-acetate, pH 7.4, 2 mM MnCl₂, and 3.2 mM NAD) with varied concentration of ICT in the absence or presence of 1 mM CIT or/and 1 mM ADP. The activity of the αβ enzyme was measured at the standard conditions but with higher concentration of Mn²⁺ (50 mM). The derived \( V_{max}, S_{0.5} \) and \( k_{cat} \) are listed in Tables 2 and 3.
Table 3. Kinetic parameters of the αβγ, αβ and αγ enzymes in the presence of positive regulators.

The V_max,ICT and S0,ICT of the αβγ, αβ and αγ enzymes in the presence of 1 mM CIT or 1 mM ADP or both were determined at the standard conditions with varied concentrations of ICT. The V_max,ICT and S0,ICT of the αβ enzyme were determined at the same conditions but with higher concentration of MnCl2 (50 mM) and varied concentrations of CIT. *A molecular mass of 80 kDa was used to calculate the mole of enzyme in heterodimeric form per mg of protein (1.25 × 10^-8 mol of dimeric enzyme/mg of protein).

| Enzyme | +CIT | +ADP | +CIT+ADP |
|--------|------|------|---------|
|        | V_max,ICT | S0,ICT | Hill coefficient for ICT | V_max,ICT | S0,ICT | Hill coefficient for ICT | V_max,ICT | S0,ICT | Hill coefficient for ICT | kcat,a | kcat/a/S0,ICT |
| αβγ    | μmol/mg/min | mM | μmol/mg/min | mM | μmol/mg/min | mM | μmol/mg/min | mM |
| αβ     | 20.7 ± 1.3 | 1.27 ± 0.06 | 1.5 ± 0.1 | 22.1 ± 0.3 | 0.868 ± 0.007 | 2.0 ± 0.1 | 21.3 ± 0.4 | 0.163 ± 0.007 | 1.5 ± 0.1 | 28.4 ± 0.5 | 174 ± 3 |
| αγ     | 11.2 ± 0.4 | 12.1 ± 0.1 | 1.1 ± 0.1 | 11.2 ± 0.3 | 12.6 ± 0.8 | 1.0 ± 0.1 | 11.9 ± 0.3 | 12.6 ± 0.8 | 1.2 ± 0.1 | 15.8 ± 0.4 | 1.25 ± 0.03 |
| αγ     | 10.0 ± 0.2 | 2.61 ± 0.12 | 1.2 ± 0.0 | 9.42 ± 0.09 | 1.69 ± 0.05 | 1.6 ± 0.1 | 13.1 ± 0.4 | 0.182 ± 0.015 | 1.0 ± 0.0 | 17.6 ± 0.5 | 96.7 ± 2.7 |

Activation and inhibition of human NAD-IDH by ATP. The previous biochemical data showed that the activities of mammalian NAD-IDHs can be negatively regulated by ATP11,20. To investigate the regulatory effect of ATP, we measured the activities of the αβγ, αβ and αγ enzymes at different concentrations of ATP in the presence or absence of CIT or both CIT and ADP. Surprisingly, our results show that ATP has both activation and inhibition effects on the activities of the αβγ and αγ enzymes but only inhibition effect on the αβ enzyme (Fig. 3). For the αβγ enzyme, ATP exhibits activation effect at low concentration with a maximum of 2.5-fold increase of the activity at [ATP] of 2 mM in the absence of any regulators, but exhibits inhibition effect at high concentration with no measurable activity at [ATP] of >3.5 mM (Fig. 3a). In the presence of CIT, the dependency of the activity on the ATP concentration displays a similar pattern with a maximum of 9-fold increase of the activity at [ATP] of 1 mM and a complete inhibition at [ATP] of >4 mM. In the presence of CIT and ADP, the αβγ enzyme has been fully activated, and thus ATP exhibits no activation effect but only inhibition effect on the activity at [ATP] of >1 mM with a complete inhibition at [ATP] of >8 mM. Similar to the αβγ enzyme, the activity of the αγ enzyme is slightly activated in the absence of CIT and significantly activated in the presence of CIT by low concentration of ATP; but is inhibited by high concentration of ATP; and additionally ATP exhibits only inhibition effect at [ATP] of >1 mM in the presence of CIT and ADP (Fig. 3c). In contrast, the activity of the αβ enzyme is completely inhibited by ATP even at low concentration in either absence or presence of CIT or CIT and ADP (Fig. 3b).

As the αβγ and αγ enzymes display higher activities in the presence of low ATP concentration, we further analyzed the effects of ATP on the kinetic parameters of these enzymes in the presence of ATP or CIT and ATP (Table 4). Compared to those in the absence of any regulators, the V_max,ICT of the αβγ enzyme is not significantly affected by ATP or CIT and ATP, but the S0,ICT is slightly decreased by 2.8-fold in the presence of ATP and substantially decreased by 12.2-fold in the presence of CIT and ATP. Similarly, the V_max,ICT of the αγ enzyme is not significantly affected by ATP or CIT or both ATP and CIT, but the S0,ICT is decreased by 1.5-fold in the presence of ATP and by 14.5-fold in the presence of CIT and ATP. Consistently, the αβγ enzyme exhibits positive cooperativity for ICT binding with a Hill coefficient of 1.8 and 1.4 in the presence of ATP and both CIT and ATP, respectively; and the αγ enzyme exhibits positive cooperativity with a Hill coefficient of 1.4 in the presence of ATP but no cooperativity in the presence of both CIT and ATP (Table 4). To avoid that the activation effect of ATP is due to contamination of ADP or hydrolyzation of ATP into ADP, we used the nonhydrolyzable ATP analogue AMP-PNP to perform the same assays, and similar activation and inhibition effects are observed (Supplementary Table S2 and Figure S1). These results indicate that ATP can activate the αβγ and αγ enzymes at low concentration but inhibit their activities at high concentration, whereas ATP exhibits only inhibition effect on the αβ enzyme. The activation of the αβγ and αγ enzymes by low concentration of ATP is in a similar manner as ADP but with a slightly weaker effect.

The γ subunit regulates both the αβ and αγ heterodimers in the holoenzyme. Our biochemical data show that the full activity of the αβγ heterotetramer requires the assembly and cooperative functions of the αβ and αγ heterodimers in both the absence and presence of activators. The previous biochemical data showed that mutation of α- Tyr126 to Phe, Ser or Glu completely abolishes the activity of the αβγ holoenzyme23. To investigate the functional roles of the β and γ subunits and the αβ and αγ heterodimers in the αβγ heterotetramer, we introduced the α- Y126F mutation into the αβ and αγ heterodimers separately, and expressed and purified the mutant αβγ and αγ γ heterodimers. The mutant αβγ and αγ γ heterotetramer was assembled by mixing the purified αβγ and αγ γ proteins with 1:1 molar ratio, and the mutant αβγ heterotetramer was assembled by mixing the purified αβγ and αγ γ proteins with 1:1 molar ratio followed by purification using gel filtration. SEC-MALS analyses indicate that introduction of the α- Y126F mutation does not affect the oligomeric states of the αβ and αγ γ proteins in solution: like the wild-type proteins, the mutant αβγ and αγ γ proteins exist as heterooctamers, and the mutant αβγ and αγ γ proteins exist as heterodimers at low concentration but as dimers of heterodimers at high concentration (Supplementary Figure S2). As expected, the
mutant $\alpha_{Y126F}\beta$ and $\alpha_{Y126F}\gamma$ heterodimers and the mutant $\alpha_{Y126F}\beta\alpha_{Y126F}\gamma$ heterotetramer have no detectable activity (Table 5). Intriguingly, the mutant $\alpha_{Y126F}\beta\alpha_{Y126F}\gamma$ heterotetramers exhibit comparable $S_{0.5,ICT}$ and about half of the catalytic efficiency compared with the wild-type holoenzyme in both the absence and presence of CIT and ADP, indicating that both $\alpha_3$ and $\alpha_\gamma$ heterodimers in the $\alpha_3\beta\gamma$ heterotetramer can be activated by the activators and contribute equally to the full activity of the holoenzyme. On the other hand, our kinetic data show that only the $\alpha_\gamma$ heterodimer alone can be activated by the activators whereas the $\alpha_3$ heterodimer alone cannot. These results led us to speculate that both $\alpha_3$ and $\alpha_\gamma$ heterodimers in the holoenzyme might be regulated by the $\gamma$ subunit. Meanwhile, our structural and biochemical studies show that the positive regulation of the $\alpha_\gamma$ heterodimer by CIT and ADP is through their binding to the allosteric site in the $\gamma$ subunit which causes conformational

Figure 3. Activation and inhibition effects of ATP. (a) The relative activity of the $\alpha_2\beta\gamma$ enzyme vs. the concentration of ATP in the absence or presence of positive regulator(s). (b) The relative activity of the $\alpha_\beta$ enzyme vs. the concentration of ATP in the absence or presence of positive regulator(s). (c) The relative activity of the $\alpha_\gamma$ enzyme vs. the concentration of ATP in the absence or presence of positive regulator(s). The activities in the absence of any regulators ($V_{\text{ab}}$) are defined as 1 and indicated by dashed lines. The activities were measured at the standard conditions with a subsaturating concentration of ICT (0.6 mM for the $\alpha_3\beta\gamma$ and $\alpha_\gamma$ enzymes and 2 mM for the $\alpha_3$ enzyme) in the absence or presence of 1 mM CIT or/and 1 mM ADP and varied concentration of ATP (0–10 mM).
changes at both the allosteric site and the active site. The \( \gamma \)-K151A mutation disrupts the structural communication between the allosteric site and the active site and thus abolishes the activation of the \( \alpha \gamma \) enzyme by CIT and ADP. Thus, we prepared the mutant \( \alpha \beta \gamma_{\text{K151A}} \) heterotetramer and carried out kinetic study. Indeed, this mutant holoenzyme has very low activity and cannot be activated by CIT and ADP (Table 5). In addition, our kinetic data show that the mutant \( \alpha \beta \gamma_{\text{K151A}} \) and \( \alpha \gamma \beta_{\text{K151A}} \) holoenzymes exhibit a Hill coefficient of 1.6–1.7 in the absence of the activators and a Hill coefficient of 1.3–1.4 in the presence of the activators, indicating that there is a cooperative binding for ICT in the absence and presence of the activators. However, the mutant \( \alpha \beta \gamma_{\text{K151A}} \) holoenzyme exhibits a Hill coefficient of 1.0 in both the absence and presence of the activators, indicating that there is no cooperative binding for ICT. These results together indicate that the \( \gamma \) subunit plays a critical role in the allosteric regulation of the holoenzyme and can regulate both the \( \alpha \beta \) and \( \alpha \gamma \) heterodimers in the \( \alpha \beta \gamma \) heterotetramer.

**Discussion**

Human NAD-IDH is an allosteric enzyme consisting of the \( \alpha \beta \) and \( \alpha \gamma \) heterodimers that are assembled into the \( \alpha \beta \gamma \) heterotetramer and further into the heterooctamer. The previous biochemical studies showed that the \( \alpha \) subunit plays the catalytic role and the \( \beta \) and \( \gamma \) subunits the regulatory role in the heterodimer. The regulatory properties of the composing \( \alpha \beta \) and \( \alpha \gamma \) heterodimers are not well studied and the exact functional roles of the \( \beta \) and \( \gamma \) subunits in the \( \alpha \beta \gamma \) heterotetramer are still elusive. In this work, we systematically characterized the enzymatic properties of the \( \alpha \beta \gamma \) heterotetramer and the \( \alpha \beta \) and \( \alpha \gamma \) heterodimers in the absence and presence of different regulators, which reveal new mechanistic insights into the function and allosteric regulation of human NAD-IDH.

Our biochemical data show that the \( \alpha \beta \gamma \) enzyme has basal activity in the absence of any regulators, and can be slightly activated by CIT or ADP alone but substantially activated by CIT and ADP together, indicating that the two activators work synergistically. The binding of CIT or/and ADP enhances the catalytic efficiency of the enzyme by decreasing the \( S_{0.5,\text{ICT}} \) but has no significant effect on the \( V_{\text{max,ICT}} \). In addition, the \( \alpha \beta \gamma \) enzyme exhibits cooperative binding for ICT both in the absence and presence of CIT or/and ADP. These data indicate that the \( \alpha \beta \gamma \) enzyme contains at least two ICT-binding sites with positive cooperativity, and CIT and ADP have distinct binding sites and can bind to the enzyme independently and simultaneously in a synergistic manner.

The \( \alpha \gamma \) heterodimer only exhibits similar kinetic properties as the \( \alpha \beta \gamma \) enzyme in both the absence and presence of the activator(s) and can also be activated by CIT or/and ADP in similar manners. In the absence of CIT and ADP, the \( S_{0.5,\text{ICT}} \) of the \( \alpha \gamma \) enzyme is decreased by 24.7-fold and the catalytic efficiency is increased by 44.8-fold which is elevated from 19.0% (in the absence of activators) to 55.6% of that of the \( \alpha \beta \gamma \) enzyme. Moreover, our kinetic data show that the \( \alpha \gamma \) enzyme exhibi

| Enzyme  | \( V_{\text{max,ICT}} \) | \( S_{0.5,\text{ICT}} \) | Hill coefficient for ICT | \( V_{\text{max,ATP}} \) | \( S_{0.5,\text{ICT}} \) | Hill coefficient for ICT | \( k_{\text{cat}} \) | \( k_{\text{cat}/S_{0.5,\text{ICT}}} \) |
|---------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| \( \alpha \beta \gamma \) | 177.0 ± 0.1 | 0.825 ± 0.021 | 1.8 ± 0.1 | 17.6 ± 0.1 | 0.193 ± 0.005 | 1.4 ± 0.0 | 23.4 ± 0.1 | 121 ± 1 |
| \( \alpha \gamma \) | 6.62 ± 0.19 | 2.98 ± 0.16 | 1.4 ± 0.1 | 8.94 ± 0.36 | 0.309 ± 0.014 | 1.1 ± 0.0 | 11.9 ± 0.5 | 38.5 ± 1.6 |

Table 4. Kinetic parameters of the \( \alpha \beta \gamma \) and \( \alpha \gamma \) enzymes in the presence of AT or both CIT and ATP. The \( V_{\text{max,ICT}} \) and \( S_{0.5,\text{ICT}} \) in the presence of 1 mM ATP or both 1 mM CIT and 1 mM ATP were determined at the standard conditions with varied concentrations of ICT. A molecular mass of 80 kDa was used to calculate the mole of enzyme in heterodimeric form per mg of protein (1.25 \( \times \) \( 10^{-4} \) mol of dimeric enzyme/mg of protein).
Table 5. Kinetic parameters of the mutant holoenzyme. The $V_{\text{max,ICT}}$ and $S_{0.5,ICT}$ in the absence or presence of both 1 mM CIT and 1 mM ADP were determined at the standard conditions with varied concentrations of ICT, except for those noted specifically. A molecular mass of 80 kDa was used to calculate the mole of enzyme in heterodimeric form per mg of protein ($1.25 \times 10^{-3}$ mol of dimeric enzyme/mg of protein). (The mutant $\alpha^{\beta\gamma\delta}$ enzyme has the $S_{0.5,\text{Ma}}$ and $S_{0.5,\text{NAD}}$ of 5.10 ± 0.46 mM and 1.54 ± 0.27 mM, respectively, which are much higher than those of the wild-type enzyme. Thus, the $V_{\text{max,ICT}}$ and $S_{0.5,ICT}$ were determined at higher concentrations of MnCl$_2$ (50 mM) and NAD (10 mM). ND: not detectable.

| Enzyme    | Specific activity | $V_{\text{max,ICT}}$ | $S_{0.5,ICT}$ | $k_{\text{cat,ICT}}$ | Hill coefficient for ICT | $V_{\text{max,ICT}}$ | $S_{0.5,ICT}$ | $k_{\text{cat,ICT}}$ | Hill coefficient for ICT |
|-----------|-------------------|----------------------|---------------|-----------------------|-------------------------|----------------------|---------------|----------------------|-------------------------|
| $\alpha^{\beta\gamma\delta}$ | 20.2 ± 0.3 | 20.0 ± 0.1 | 2.35 ± 0.05 | 11.36 ± 0.04 | 2.0 ± 0.1 | 21.3 ± 0.4 | 0.163 ± 0.007 | 174 ± 3 | 1.5 ± 0.1 |
| $\alpha^{\beta\gamma\delta}$ | 0 | ND | ND | ND | ND | ND | ND | ND | ND |
| $\alpha^{\beta\gamma\delta}$ | 0 | ND | ND | ND | ND | ND | ND | ND | ND |
| $\alpha^{\beta\gamma\delta}$ | 8.45 ± 0.14 | 8.47 ± 0.42 | 2.12 ± 0.13 | 5.32 ± 0.13 | 1.6 ± 0.1 | 8.43 ± 0.32 | 0.148 ± 0.005 | 75.9 ± 5.1 | 1.4 ± 0.1 |
| $\alpha^{\beta\gamma\delta}$ | 9.00 ± 0.15 | 9.07 ± 0.39 | 2.41 ± 0.12 | 5.02 ± 0.12 | 1.7 ± 0.1 | 9.12 ± 0.39 | 0.129 ± 0.006 | 94.2 ± 3.0 | 1.3 ± 0.1 |
| $\alpha^{\beta\gamma\delta}$ | 1.76 ± 0.11 | 8.24 ± 0.59 | 21.9 ± 1.1 | 0.501 ± 0.035 | 1.0 ± 0.1 | 8.02 ± 0.42 | 17.6 ± 1.5 | 0.607 ± 0.032 | 1.0 ± 0.1 |

The $\alpha^{\beta\gamma\delta}$ heterodimer also contributes to the full activity of the holoenzyme in both the absence and presence of the activators, and can be activated by CIT and ADP through the $\gamma$ subunit. These data together suggest that the $\beta$ subunit plays a structural role in the $\alpha^{\beta\gamma\delta}$ heterotetramer to facilitate the assembly and thus ensure the full activity of the holoenzyme. The previous biochemical data showed that several residues of the $\beta$ subunit, including $\beta$-Arg99, $\beta$-Tyr137, $\beta$-Asp192 and $\beta$-Asp217, play important roles in the function and regulation of the $\alpha^{\beta\gamma}$ enzyme.\cite{13,15,16,23}. Our mutagenesis and biochemical data show that although these mutations have no effect on the basal activity of the $\alpha$'s enzyme, some of them at the heterodimer interface have some effects on the activity of the $\alpha^{\beta\gamma}$ enzyme in the absence and particularly in the presence of the activators (Ma et al., unpublished data). It is very likely that the $\beta$ subunit is involved in the heterodimer-heterodimer and/or heterotetramer-heterotetramer interfaces in the holoenzyme, and the conformational changes at the allosteric site in the $\gamma$ subunit induced by the binding of activators are transmitted to the active sites in the $\alpha^{\beta\gamma}$ and $\beta^{\gamma}$ heterodimers via the interfaces, and thus mutations of the $\beta$ subunit at the heterodimer interface would affect the cooperative function(s) of the two heterodimers and/or heterotetramers and hence compromise the full activity of the holoenzyme.

Surprisingly, our biochemical data show that ATP has both activation and inhibition effects on the $\alpha^{\beta\gamma\delta}$ and $\alpha^{\beta\gamma}$ enzymes dependent on the concentration but only inhibition effect on the $\alpha^{\beta}$ enzyme, which are different from the previous biochemical data showing that ATP only inhibits the activity of bovine heart NAD-IDH.\cite{11} In the absence of any activators or the presence of CIT, ATP can activate the $\alpha^{\beta\gamma\delta}$ and $\alpha^{\beta\gamma}$ enzymes at low concentration but inhibit their activities at high concentration. In the presence of both CIT and ADP, ATP exhibits only inhibition effect on the activities as the enzymes have been fully activated. Moreover, our kinetic data show that the activation effect of ATP is exerted in a similar manner as ADP. These results lead us to speculate that there might be two different binding sites for ATP in the $\alpha^{\beta\gamma\delta}$ and $\alpha^{\beta\gamma}$ enzymes. One binding site might be the same site that ATP binds in the $\alpha^{\beta\gamma\delta}$ and $\alpha^{\beta\gamma}$ enzymes. The other binding site might be at the active site, as suggested earlier,\cite{11,27} and the binding of ATP to this site would compete with the NAD binding and thus inhibits the activity. It is also possible that high concentration of ATP may compete for binding with the metal ion and thus confers inhibition on the activity. More biochemical and structural studies are needed to dissect the molecular mechanism of the dual effect of ATP.

Taken together, our biochemical data indicate that the $\alpha^{\beta}$ and $\alpha^{\beta\gamma}$ enzymes alone have considerable basal activities but exhibit different enzymatic properties: the $\alpha^{\beta}$ enzyme shares very similar kinetic characteristics and allosteric regulation patterns as the $\alpha^{\beta\gamma}$ enzyme, whereas the $\alpha^{\beta}$ enzyme does not. Both the $\alpha^{\beta}$ and $\alpha^{\beta\gamma}$ heterotetramers in the $\alpha^{\beta\gamma\delta}$ heterotetramer contribute equally to the full activity of the holoenzyme in both the absence and presence of the activators, and the positive regulation of the $\alpha^{\beta}$ and $\alpha^{\beta\gamma}$ heterodimers in the $\alpha^{\beta\gamma\delta}$ heterotetramer is through the $\gamma$ subunit but not the $\beta$ subunit. These results together demonstrate that the $\alpha$ subunits play the catalytic role, the $\gamma$ subunit the regulatory role, and the $\beta$ subunit the structural role in the $\alpha^{\beta\gamma\delta}$ heterotetramer. Further biochemical and structural studies of the $\alpha^{\beta\gamma\delta}$ heterotetramer will provide more insights into the molecular basis of the specific functional roles of the $\beta$ and $\gamma$ subunits and the $\alpha^{\beta}$ and $\alpha^{\beta\gamma}$ heterodimers in the $\alpha^{\beta\gamma\delta}$ heterotetramer and reveal the catalytic and regulatory mechanisms of the $\alpha^{\beta\gamma\delta}$ heterotetramer.

Methods

Cloning, expression, and purification. The $\alpha^{\beta}$ and $\alpha^{\beta\gamma}$ heterodimers and the $\alpha^{\beta\gamma\delta}$ heterotetramer of human NAD-IDH were prepared using a method different from that described previously.\cite{22} Human IDH3A, IDH3B (isoform 2), and IDH3G genes in vector pReceiver-B01/B02 were purchased from FulenGen (China). The N-terminal 27, 34 and 39 residues of the $\alpha$, $\beta$ and $\gamma$ subunits, respectively, which are the signal peptides for their translocation into the mitochondria,\cite{40} were removed during construction. The DNA fragments of the $\alpha$, $\beta$ and
γ subunits were individually cloned into the co-expression vector pQlinkN with the C-terminals of the β and γ subunits attached with or without a TEV protease cleavage site and a His6 tag, which were then used to construct the pQlinkN-α-β-γ-tev-His6, pQlinkN-α-γ-tev-His6, pQlinkN-α-β, and pQlinkN-α-γ plasmids, and subsequently the pQlinkN-α-γ-β-γ-tev-His6 plasmid, following the pQlink cloning procedure41. The pQlinkN-α-β-γ-tev-His6, and pQlinkN-α-γ-β-γ-tev-His6 plasmids were transformed into E. coli BL21(DE3) Codon-Plus strain (Novagen) for expressions of the αβδ and αγδ heterodimers, and the pQlinkN-α-γ-α-β-γ-tev-His6 plasmid for expression of the αβδγ heterotetramer. When the culture of the transformed cells reached an OD₆₀₀ of 0.4–0.6, the protein expression was induced by 0.4 mM IPTG for 20 hr at 23 °C. The bacterial cells were harvested, resuspended, and sonicated on ice in a lysis buffer (50 mM HEPEs-Na, pH 7.4, 200 mM NaCl, 0.2 mM MnCl₂, 10% (w/v) glyceral, and 7.2 mM β-ME) supplemented with 1 mM PMSF. The target protein was firstly purified by affinity chromatography using a Ni-NTA column (Qiagen) with the lysis buffer supplemented with 20 mM and 200 mM imidazole serving as the washing buffer and elution buffer, respectively. The elution fraction was dialyzed against the lysis buffer overnight to lower the concentration of imidazole to <10 mM; meanwhile, the His₆-tag was cleaved by TEV protease. The protein mixture was reloaded on a Ni-NTA column, which was then washed with the lysis buffer supplemented with 10 mM imidazole. The flow-through fraction containing the target protein was concentrated by centrifugation using an Amicon Ultra-4 centrifugal filter unit with Ultracel-30 membrane (Millipore), and then purified by gel filtration using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with the storage buffer (10 mM Tris-acetate, pH 7.4, 200 mM NaCl, and 5 mM β-ME). As the yield of the co-expressed αβδγ heterotetramer was much lower than those of the αβδ and αγδ heterodimers, we assembled the αβδγ heterotetramer by either co-purifying the separately expressed αβδ and αγδ heterodimers using a combination of affinity chromatography and gel filtration, or mixing the purified αβδ and αγδ heterodimers at 1:1 molar ratio overnight followed by purification using gel filtration chromatography. Both methods produced the αβδγ heterotetramer with much higher yields. Purities of the protein samples were assessed by 12% SDS-PAGE with Coomassie blue staining. Concentrations of the proteins were determined using the BCA Protein Assay Kit (Thermo Scientific). Mutants of the αβδ and αγδ heterodimers containing point mutations in the αβδ and γ subunits were constructed using the QuikChange® Site-Directed Mutagenesis kit (Strategene). Expression and purification of the mutants were carried out the same as for the wild-type proteins.

SEC-MALS analysis. The purity, molecular mass and size distribution of the proteins were analyzed by an analytical light scattering instrument (SEC-MALS) consisting of an Agilent 1260 Infinity Isocratic Liquid Chromatography System, a Wyatt Dawn Heleos II Multi-Angle Light Scattering Detector (Wyatt Technology) and a Wyatt Optilab T-rEX Refractive Index Detector (Wyatt Technology). Analytical size exclusion chromatography was performed at room temperature using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with a mobile phase containing 10 mM HEPEs, pH 7.4, 200 mM NaCl, and 5 mM β-ME. 100 μl protein sample at concentration of 2 mg/ml or 12 mg/ml was injected into the column and eluted at a flow rate of 0.4 ml/min. The column effluent was monitored in-line with three detectors that simultaneously monitor the UV absorption, light scattering and refractive index. The measurements were analyzed using the ASTRA software (Wyatt Technology) to determine the molecular mass of the protein42.

Enzymatic activity assay. The activities of the αβδγ, αβδ and αγδ enzymes were determined by monitoring the time-dependent formation of NADH at 340 nm (ε = 6220 M⁻¹ cm⁻¹) using a Coulter DU 800 Spectrophotometer (Beckman) at 25 °C. The standard reaction solution (1 ml) consisted of 33 mM Tris-acetate, 2 mM enzyme, 80 mM DL-isocitrate, 2 mM MnCl₂, and 3.2 mM NAD. The catalytic reaction was initiated by addition of NAD. All the experiments were performed in three independent measurements and the values were the averages of the three measurements with the standard errors.

For determination of the effect of ATP, the activities of the enzymes were measured at the standard conditions except for a subsaturating concentration of ICT (0.6 mM for the αβδγ and αγδ enzymes and 2 mM for the αβδ enzyme) and varied concentrations of ATP (0–10 mM).

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Author Contributions
T.M. carried out the cloning, protein purification and biochemical studies. Y.P. and W.H. participated in the initial cloning, expression and purification experiments. Y.L. participated in the biochemical studies. J.D. conceived the study, participated in the designs and data analyses of all experiments, and wrote the manuscript.

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