Molecular mechanism of the allosteric regulation of the αγ heterodimer of human NAD-dependent isocitrate dehydrogenase

Tengfei Ma, Yingjie Peng, Wei Huang & Jianping Ding

Human NAD-dependent isocitrate dehydrogenase catalyzes the decarboxylation of isocitrate (ICT) into α-ketoglutarate in the Krebs cycle. It exists as the αβγ heterotetramer composed of the αβ and αγ heterodimers. Previously, we have demonstrated biochemically that the αβγ heterotetramer and αγ heterodimer can be allosterically activated by citrate (CIT) and ADP. In this work, we report the crystal structures of the αγ heterodimer with the γ subunit bound without or with different activators. Structural analyses show that CIT, ADP and Mg2+ bind adjacent to each other at the allosteric site. The CIT binding induces conformational changes at the allosteric site, which are transmitted to the active site through the heterodimer interface, leading to stabilization of the ICT binding at the active site and thus activation of the enzyme. The ADP binding induces no further conformational changes but enhances the CIT binding through Mg2+-mediated interactions, yielding a synergistic activation effect. ICT can also bind to the CIT-binding subsite, which induces similar conformational changes but exhibits a weaker activation effect. The functional roles of the key residues are verified by mutagenesis, kinetic and structural studies. Our structural and functional data together reveal the molecular mechanism of the allosteric regulation of the αγ heterodimer.

Isocitrate dehydrogenases (IDHs) are a family of enzymes that catalyze the oxidative decarboxylation of isocitrate (ICT) into α-ketoglutarate (α-KG) using NADP or NAD as coenzyme. Most bacteria and archaea contain only NADP-dependent IDHs (NADP-IDHs, EC 1.1.1.42) in the cytosol, which carry out the catalytic reaction in the Krebs or tricarboxylic acid (TCA) cycle. Eukaryotes contain both NADP-IDHs and NAD-dependent IDHs (NAD-IDHs, EC 1.1.1.41). The eukaryotic NAD-IDHs, located in the mitochondria, exert the catalytic activity in the Krebs cycle. The eukaryotic NAD-IDHs, located in the cytosol, mitochondria and peroxisomes, are demonstrated to play important roles in cellular defense against oxidative damage, detoxification of reactive oxygen species, and synthesis of fat and cholesterol. In particular, human cytosolic and mitochondrial NADP-IDHs (also called IDH1 and IDH2) have been implicated in oncogenesis of tumors. Mutations of human IDH1 and IDH2 have been identified in multiple types of tumors and the mutant proteins confer new function to convert α-KG into 2-hydroxyglutarate (2-HG) whose accumulation can result in altered metabolism and epigenetic dysregulation, leading to genesis and development of cancers.

The molecular mechanisms of the function and catalytic reaction of NADP-IDHs have been extensively studied at the biochemical and structural levels. The crystal structures of NADP-IDHs from various species, including E. coli NADP-IDH (EcIDH), porcine mitochondrial NADP-IDH (PmIDH) and human cytosolic NADP-IDH...
activation of the enzyme. The ADP binding does not induce further conformational changes but enhances the site through the heterodimer interface, leading to stabilization of the ICT binding at the active site and thus CIT binding induces significant conformational changes at the allosteric site, which are transmitted to the active site and coenzyme NAD. The crystal structure of *Saccharomyces cerevisiae* NAD-IDH has been reported, which shows that the binding of CIT and AMP at the allosteric site could induce conformational changes of the active site and thus enhances the binding affinity for ICT.

Mammalian NAD-IDHs are even more complex than yeast NAD-IDH. These enzymes are composed of three types of subunits in the ratio of 2α:1β:1γ, which share about 40–52% sequence identity. The α and β subunits form one heterodimer (αβ) and the α and γ subunits form another heterodimer (αγ), which are assembled into a heterotetramer (αβγ) and further into a heteroctamer (the heterotetramer and heterooctamer are sometimes called holoenzyme). The previous biochemical data showed that the α subunit is essential for the catalytic activity whereas the β and γ subunits play regulatory roles in the αβγ heterotetramer, and the activity of the αγγ heterotetramer is positively regulated by CIT and ADP but inhibited by ATP and NADH. It was suggested that the αβγ heterotetramer contains two binding sites for each ligand, including Mn2+, ICT, NAD, and ADP, which are probably located at the interfaces of the α and β subunits and the α and γ subunits (αβγ:1:1 and αβγ:1:2). In our previous work, we studied systematically the enzymatic properties of the αβγ heterotetramer and the αβ and αγ heterodimers of human NAD-IDH (also called IDH3) and the specific functions of the β and γ subunits in the αβγ heterotetramer. Our biochemical data demonstrate that the αγ heterodimer exhibits similar enzymatic properties as the αβγ heterotetramer and can be positively regulated by CIT and ADP, whereas the αβ heterodimer has only basal activity and cannot be regulated. Furthermore, we show that in the αβγ heterotetramer, the γ subunit plays the regulatory role to activate the holoenzyme and the β subunit plays the structural role to facilitate the assembly and ensure the full activity of the holoenzyme. However, the molecular basis for the assembly of the αβγ heterotetramer and the molecular mechanism of the allosteric regulation of the αγ heterodimer and the αβγ heterotetramer are still elusive.

In this work, to investigate how the γ subunit plays the regulatory role to activate the αγ heterodimer, we determined the crystal structures of the αγ heterodimer with the γ subunit bound without or with the positive regulators CIT and ADP, and carried out detailed mutagenesis and kinetic studies to validate the functional roles of the key residues involved in the binding of the regulators and the conformational changes. We found that the CIT binding induces significant conformational changes at the allosteric site, which are transmitted to the active site through the heterodimer interface, leading to stabilization of the ICT binding at the active site and thus activation of the enzyme. The ADP binding does not induce further conformational changes but enhances the CIT binding through Mg2+-mediated interactions, yielding a synergistic activation effect. Intriguingly, we also found that ICT can bind to the CIT-binding subsite, which induces similar conformational changes but exhibits a weaker activation effect. Our structural and biochemical data together reveal the molecular basis for the interplay of different regulators and the molecular mechanism of the allosteric regulation of the αγ heterodimer.

### Results

#### Overall structure of the αγ heterodimer.

The preparation and biochemical characterization of the αγ heterodimer of human NAD-IDH were described in details previously. The crystal structures of the αγ heterodimer with the γ subunit bound without any regulators (αγγγ), and bound with a Mg2+ and a CIT (αγγγ:1:1:1), with a Mg2+, a CIT and an ADP (αγγγ:1:1:1:1), and with a Mg2+, an ICT and an ADP (αγγγ:1:1:1:1), and the αγγγ mutant bound with a Mg2+, a CIT, and an ADP (αγγγ:1:1:1:1) were determined at 2.8 Å, 2.3 Å, 2.65 Å, 2.8 Å, and 2.5 Å resolution, respectively (Table 1). These structures all belong to space group P3121 with one asymmetric unit containing one αγ heterodimer. In all of these structures, the polypeptide chains of both the α and γ subunits are well defined with high-quality electron density except for a few N-terminal and/or C-terminal residues. At the active site and/or the allosteric site, there was strong electron density which was interpreted as Mg2+ due to the presence of Mg2+ (>0.2 mM) in the crystallization solutions (Supplementary Figure S1). In the ligand-bound structures, there was evident electron density at the allosteric site matching the corresponding ligand(s) very well (Supplementary Figure S1). The αγ heterodimer in different ligand-bound structures shows very similar overall structure with RMSD of <1.2 Å for over 650 Cα atoms when compared pair-wisely (Supplementary Table S1). However, there were notable conformational differences at the allosteric site, the active site and the heterodimer interface upon the binding of CIT (see discussion later).

Similar to NADP-IDHs and yeast NAD-IDH, both the α and γ subunits consist of 10 α-helices (α1–α10) and 12 β-strands (31–312) which fold into three domains: a large domain assuming a typical Rossmann fold, a small domain assuming an αβ sandwich fold, and a clamp domain consisting of two anti-parallel β-strands (Fig. 1 and Supplementary Figure S2). The α and γ subunits form a compact heterodimer with a pseudo two-fold symmetry and the heterodimer interface is mainly mediated by extensive hydrophilic and hydrophobic interactions between the α6 and α7 helices of the small domains which form a four-helix bundle, and between the α36 and α37 strands of the clamp domains which form a four-stranded β-sheet (Fig. 1a). The active site is located in the cleft formed by the large and small domains of the α subunit and the small domain of the γ subunit, and comprises of the binding sites for the metal ion, substrate ICT and coenzyme NAD. The allosteric site is located in the cleft formed by the large and small domains of the γ subunit and the small domain of the α subunit, and comprises of the binding sites for the metal ion and activators CIT and ADP.
It is noteworthy that in all of these structures, two αγ heterodimers related by the crystallographic two-fold axis form a dimer of heterodimer or a heterotetramer via the four-stranded β-sheets of the clasp domains, which form an eight-stranded β-barrel. This is in agreement with our previous biochemical data showing that the αγ protein exists mainly as a heterodimer at low concentration in solution but as a dimer of heterodimer at high concentration40. As the dimer of the αγ heterodimer is formed in a similar manner as the IDH1/IDH2 heterotetramer of yeast NAD-IDH21, it is possible that the αγ heterotetramer might mimic the α2βγ heterotetramer of human NAD-IDH.

Binding of CIT induces conformational changes at the allosteric site. The previous biochemical studies showed that mammalian NAD-IDHs can be activated by CIT through decreasing the $S_{0.5,ICT}$ value32. Our biochemical data have shown that the αγ heterodimer can be activated by CIT in a manner similar to the α2βγ heterotetramer40. To investigate the regulatory mechanism of CIT activation, we determined the αMgγ and αMgγMg+CIT structures (Table 1). Comparison of the two structures shows that the CIT binding does not cause notable conformational change in the overall structure (Supplementary Table S1), but induces significant conformational changes at the allosteric site, the active site, and the heterodimer interface (Fig. 2a).

In the αMgγ structure, there are no metal ion and ligand bound at the allosteric site and the key residues composing the allosteric site are stabilized by several hydrogen bonds (Fig. 2b, left panel). Specifically, the side chain of Tyr135G (residues of the α and γ subunits are superscripted by “A” and “G”, respectively) forms hydrogen

| Structure              | αMgγ | αMgγ+CIT | αMgγ+CIT+ADP | αMgγ+ICT+ADP | αγK151A+ICT+ADP |
|------------------------|------|---------|-------------|--------------|-----------------|
| Diffraction data       |      |         |             |              |                 |
| Wavelength (Å)         | 1.0000 | 1.0000 | 1.0000 | 0.9792 | 0.9792 |
| Space group            | P3,21 | P3,21   | P3,21       | P3,21        | P3,21           |
| a (Å)                  | 118.4 | 112.0   | 104.9       | 111.2        | 114.3           |
| b (Å)                  | 118.4 | 112.0   | 104.9       | 111.2        | 114.3           |
| c (Å)                  | 143.2 | 145.0   | 146.3       | 145.5        | 143.9           |
| Resolution (Å)         | 19.71–2.80 (2.90–2.80) | 50.0–2.30 (2.38–2.30) | 50.0–2.65 (2.74–2.65) | 50.0–2.80 (2.90–2.80) | 50.0–2.50 (2.59–2.50) |
| Observed reflections   | 309,587 | 906,555 | 250,203 | 72,179 | 187,289 |
| Unique reflections (I/σ(I)>0) | 28,718 | 46,471 | 25,700 | 25,878 | 38,210 |
| Average redundancy     | 10.8 (10.6) | 19.5 (14.4) | 9.1 (9.2) | 2.8 (2.8) | 4.9 (5.0) |
| Average I/σ(I)         | 9.6 (4.4) | 53.7 (3.9) | 22.4 (4.4) | 14.5 (2.5) | 23.4 (2.9) |
| Completeness (%)       | 98.9 (100.0) | 98.4 (86.6) | 99.9 (100.0) | 98.1 (99.6) | 99.9 (99.9) |
| Rmerge (%)             | 15.6 (48.2) | 6.2 (45.8) | 10.7 (53.7) | 8.0 (49.8) | 7.5 (45.7) |

Table 1. Statistics of X-ray diffraction data and structure refinement. *Numbers in parentheses refer to the highest resolution shell. $R_{merge} = \sum_{hkl} \sum_{i} |I_{i}(hkl) - \langle I(hkl) \rangle| / \sum_{i} \sum_{hkl} I_{i}(hkl)$. R factor = $\sum|F_{o}| - |F_{c}| / \sum|F_{o}|$.  

It is noteworthy that in all of these structures, two αγ heterodimers related by the crystallographic two-fold axis form a dimer of heterodimer or a heterotetramer via the four-stranded β-sheets of the clasp domains, which form an eight-stranded β-barrel. This is in agreement with our previous biochemical data showing that the αγ protein exists mainly as a heterodimer at low concentration in solution but as a dimer of heterodimer at high concentration40. As the dimer of the αγ heterodimer is formed in a similar manner as the IDH1/IDH2 heterotetramer of yeast NAD-IDH21, it is possible that the αγ heterotetramer might mimic the αβγ heterotetramer of human NAD-IDH.

Binding of CIT induces conformational changes at the allosteric site. The previous biochemical studies showed that mammalian NAD-IDHs can be activated by CIT through decreasing the $S_{0.5,ICT}$ value32. Our biochemical data have shown that the αγ heterodimer can be activated by CIT in a manner similar to the αβγ heterotetramer40. To investigate the regulatory mechanism of CIT activation, we determined the αMgγ and αMgγMg+CIT structures (Table 1). Comparison of the two structures shows that the CIT binding does not cause notable conformational change in the overall structure (Supplementary Table S1), but induces significant conformational changes at the allosteric site, the active site, and the heterodimer interface (Fig. 2a).

In the αMgγ structure, there are no metal ion and ligand bound at the allosteric site and the key residues composing the allosteric site are stabilized by several hydrogen bonds (Fig. 2b, left panel). Specifically, the side chain of Tyr135G (residues of the α and γ subunits are superscripted by “A” and “G”, respectively) forms hydrogen
activity. In both the molecules (Supplementary Figure S1c).

Thr269G; and the side chain of Asn78G also points away from the CIT-binding subsite to form hydrogen bonds between the side chains of Arg97G and Tyr135G and between the side chains of Arg272G and Asn78G in the allosteric site; and in the binding induces the conformational changes of Asn78G and Arg272G, both of which make coordination bonds of Asn78G, Asn93G and Arg272G are also rotated towards the CIT-binding subsite to make hydrogen-bonding interactions with the surrounding residues and forms a coordination bond with the Mg\(^{2+}\) structure are disrupted. Meanwhile, the side chain of Tyr135G is rotated by about 30° with the hydroxyl group away from the CIT-binding subsite to interact with the side chain of Asn78G and the main-chain carbonyl of Thr271G and Asn273G.

In the \(\alpha\)\(\gamma\) heterodimer, the \(\gamma\) subunit is likely dependent on the binding of CIT, consistent with the observation that the CIT binding induces the conformational changes of Asn78G and Arg272G, both of which make coordination bonds with the Mg\(^{2+}\) and form part of the Mg\(^{2+}\) binding subsite (Supplementary Figure S1c).

**Binding of CIT also induces conformational changes at the active site.** Our biochemical data have shown that similar to the \(\alpha\beta\gamma\) heterotetramer, the \(\alpha\gamma\) heterodimer requires a divalent metal ion for the enzymatic activity. In both the \(\alpha\)\(\beta\gamma\) and \(\alpha\)\(\beta\)\(\gamma\)\(\beta\)\(\gamma\) structures, there is a Mg\(^{2+}\) bound at the active site, which is coordinated by Asp230\(\alpha\), Asp234\(\alpha\), Asp215\(\alpha\) and water molecules (Supplementary Figure S1a,b). The functional roles of Asp230\(\alpha\), Asp234\(\alpha\) and Asp215\(\alpha\) of human NAD-IDH have been validated by the previous mutagenesis and
Figure 2. Binding of CIT induces conformational changes at the allosteric site, the active site, and the heterodimer interface. (a) Comparison of the $\alpha^{\text{Mg}}$ and $\alpha^{\text{Mg}}$-Mg+CIT structures. The $\alpha$ and $\gamma$ subunits in the $\alpha^{\text{Mg}}$ structure are colored in lemon and cyan, respectively, and these in the $\alpha^{\text{Mg}}$-Mg+CIT structure in magenta and orange, respectively. Major conformational changes are observed at the allosteric site, the active site, and the $\beta_5$-$\beta_6$ loop, the $\alpha_7$ helix and the $\beta_7$ strand at the heterodimer interface. The orientations of the $\alpha_6$ and $\alpha_7$ helices in both subunits are indicated with dashed arrows. Some key residues involved in the conformational changes are shown with side chains. The zoom-in panel on the right top shows the conformational changes of the $\beta_7\text{A}$ and $\beta_7\text{G}$ strands. For clarity, only the hydrogen-bonding interactions between the main chains are shown and the side chains are omitted. The zoom-in panel in the right bottom shows the loop-to-helix transition of the N-terminal region of the $\alpha_7\text{A}$ and $\alpha_7\text{G}$ helices. (b) Structure of the allosteric site in the $\alpha^{\text{Mg}}$.
Glu132G forms a hydrogen bond each with the main-chain amine and the side chain of Thr154G; the main-chain
α-subunit, the side chain of among residues of these structure elements (Fig. 2e,f left panel). Specifically, in the
αMgCIT structure, the side chain of Tyr126G forms a hydrogen bond with the side chain of Arg119G, and the side chain of Asp230G forms two coordination bonds with the Mg2+; whereas in the αMg7 CIT structure, the side chain of Tyr126G is rotated by about 30° with the hydroxyl group moving about 7 Å to form a hydrogen bond with the side chain of Asp230G, and concurrently the side chain of Asp230G is rotated towards Tyr126G and additionally forms two coordination bonds with the Mg2+ via the side-chain carboxyl and main-chain carboxyl groups. Further structural comparison shows that the side-chain conformations of Tyr126G and Asp230G in the αMg7 CIT structure are similar to those in the ICT-bound PmIDH and HcIDH structures, in which the equivalent residues (Tyr140 and Asp275 in PmIDH and Tyr139 and Asp275 in HcIDH) form a hydrogen bond with each other and additionally make hydrogen-bonding interactions with ICT (Supplementary Figure S3). This implies that the side chains of Tyr126G and Asp230G in the αMg7 CIT structure are in proper conformations to bind ICT. These results indicate that the CIT binding at the allosteric site induces marked conformational changes of two important residues at the active site, which could facilitate the substrate binding. This is consistent with the biochemical data showing that the S50.5 values for Mn2+ and/or ICT, and thus decrease the activity. Sequence comparison shows that Asp230G and Asp234G of human NAD-IDH are strictly conserved in NADP-IDHs and the catalytic subunit of other NAD-IDHs, but are replaced by Asn or Thr in the regulatory subunits of NAD-IDHs; and Asp215G is strictly conserved in both the catalytic and regulatory subunits of NAD-IDHs and in NADP-IDHs (Supplementary Figure S2). The corresponding residues in NADP-IDHs are also involved in the binding of the metal ion and some of them in the binding of ICT, and thus play vital roles in the catalytic reaction.

Sequence comparison of human NAD-IDH with NADP-IDHs and other NAD-IDHs shows that the key residues involved in the binding of ICT at the active site are strictly conserved, including Thr74G, Ser82G, Arg88G, Arg98G, Arg119G, Tyr126G, Asp230G, Lys182G and Asp215G (Supplementary Figure S2). Very intriguingly, comparison of the αMg7 and αMg7 CIT structures shows that although most of the residues composing the active site exhibit no significant conformational differences, Tyr126G and Asp230G undergo marked conformational changes (Fig. 2d). In the αMg7 structure, the side chain of Tyr126G forms a hydrogen bond with the side chain of Arg119G and the side chain of Asp230G forms two coordination bonds with the Mg2+; whereas in the αMg7 CIT structure, the side chain of Tyr126G is rotated by about 30° with the hydroxyl group moving about 7 Å to form a hydrogen bond with the side chain of Asp230G, and concurrently the side chain of Asp230G is rotated towards Tyr126G and additionally forms two coordination bonds with the Mg2+ via the side-chain carboxyl and main-chain carboxyl groups. Further structural comparison shows that the side-chain conformations of Tyr126G and Asp230G in the αMg7 CIT structure are similar to those in the ICT-bound PmIDH and HcIDH structures, in which the equivalent residues (Tyr140 and Asp275 in PmIDH and Tyr139 and Asp275 in HcIDH) form a hydrogen bond with each other and additionally make hydrogen-bonding interactions with ICT (Supplementary Figure S3). This implies that the side chains of Tyr126G and Asp230G in the αMg7 CIT structure are in proper conformations to bind ICT. These results indicate that the CIT binding at the allosteric site induces marked conformational changes of two important residues at the active site, which could facilitate the substrate binding. This is consistent with the biochemical data showing that the S50.5 values for Mn2+ in the α7 CIT structure, the interactions between CIT and the surrounding residues and the Mg2+ are indicated with dashed lines. (c) Comparison of the allosteric site in the αMg7 and αMg7 CIT structures. (d) Comparison of the active site in the αMg7 and αMg7 CIT structures. (e) A schematic diagram showing the hydrogen-bonding interactions among the 35–36 loop, the α7 helix, the β strand, and the α5 helix in the αMg7 and αMg7 CIT structures. The interactions in the αMg7 structure are indicated with green lines; these disrupted upon the CIT binding are indicated with dashed green lines and the newly formed interactions with red lines. (f) Structure of the heterodimer interface in the αMg7 (left panel) and αMg7 CIT (right panel) structures. For clarity, only the hydrogen-bonding interactions altered upon the CIT binding are indicated with dashed lines.

Biochemical data showing that mutations of these residues would substantially increase the S50.5 values for Mn2+ and/or ICT, and thus decrease the activity. Sequence comparison shows that Asp230G and Asp234G of human NAD-IDH are strictly conserved in NADP-IDHs and the catalytic subunit of other NAD-IDHs, but are replaced by Asn or Thr in the regulatory subunits of NAD-IDHs; and Asp215G is strictly conserved in both the catalytic and regulatory subunits of NAD-IDHs and in NADP-IDHs (Supplementary Figure S2). The corresponding residues in NADP-IDHs are also involved in the binding of the metal ion and some of them in the binding of ICT, and thus play vital roles in the catalytic reaction.

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Conformational changes at the allosteric site are transmitted to the active site via the heterodimer interface. To understand the molecular basis for the communication between the allosteric site and the active site, we carried out a detailed comparison of the αMg7 and αMg7 CIT structures and found that upon the CIT binding, in addition to the conformational changes at the allosteric site and the active site, several structural elements at the heterodimeric interface, especially the 35–36 loop and the α7 helix of the small domain, and the β strand of the clasp domain in both the α and γ subunits, also undergo substantial conformational changes in a pseudo symmetric manner (Fig. 2a). In the absence of CIT (the αMγ structure), in both subunits, the N-terminal region of the α7 helix adopts a loop conformation and there are a number of hydrogen-bonding interactions among residues of these structure elements (Fig. 2e,f left panel). Specifically, in the γ-subunit, the side chain of Glu132G forms a hydrogen bond each with the main-chain amine and the side chain of Thr154G; the main-chain amine of Gly133G forms a hydrogen bond with the main-chain carbonyl of Ile152G; the main-chain amine of Glu134G forms a hydrogen bond with the main-chain carbonyl of Asn235G; and the main-chain amine of Leu138G forms a hydrogen bond with the main-chain carbonyl of Val150G; however, there is no hydrogen-bonding interaction between the α7D helix and the β7 strand. Similarly, in the α-subunit, the side chain of Glu123G forms a hydrogen bond each with the main-chain amine and the side chain of Thr145G; the main-chain amine of Gly124G forms a hydrogen bond with the main-chain carbonyl of Leu143G; the main-chain amine of Glu125G forms a hydrogen bond with the main-chain carbonyl of Asn226G; and the main-chain amine of Ile129G forms a hydrogen bond with the main-chain carbonyl of Ile141G; but there is no hydrogen-bonding interaction between the α7A helix and the β7 strand.

Upon the CIT binding (the αMg7 CIT structure), in both subunits, the C-terminal region of the 35–36 loop adopts an alternative conformation; the N-terminal region of the α7 helix assumes an α-helical conformation to form a long α7 helix; and the β7 strand bends towards the 35–36 loop and the α7 helix (the Cα atoms of Ser149G-Lys151G and Ser149G-Lys152G are shifted by about 1–2 Å) (Fig. 2a). Consequently, some of the hydrogen-bonding interactions among these structure elements in the αMγ structure are disrupted, and a new and more intensive network of hydrogen-bonding interactions is established (Fig. 2e,f right panel). Specifically, in
the γ-subunit, accompanying the conformational changes of Arg97G and Tyr135G, the hydrogen bonds between Glu134G and Asn223G and between Leu138G and Leu150G are disrupted. On the other hand, the main chain of Gly133G forms a new hydrogen bond with the main chain of Ile152G; the side chain of Glu134G is inserted into the space formerly occupied by the N-terminal loop of the αβ helix and forms a new hydrogen bond with the main-chain amine of Leu236G; the side chain of Lys151G forms two hydrogen bonds with the side chains of Tyr237G and Asp190G, and the side chains of Tyr237G and Asp190G also form a hydrogen bond with each other. Similarly, in the α subunit, the hydrogen bonds between Glu125G and Asn226G and between Ile129G and Ile141G are disrupted. Meanwhile, the main chain of Gly124G gains a hydrogen bond with the main chain of Leu143G; the side chain of Glu125G is inserted into the space formerly occupied by the N-terminal loop of the αβ helix and forms a hydrogen bond with the main-chain amine of Leu227G; the side chain of Lys142G forms hydrogen bonds with the side chains of Tyr228G and Asp181G, and the side chains of Tyr228G and Asp181G form a hydrogen bond with each other. Consequently, the side chain of Tyr126G is rotated towards the active site and assumes a proper position to interact with ICT. The conformational changes of these structure elements at the heterodimer interface and the alterations of the hydrogen-bonding interactions among the residues associated with the CIT binding provide the molecular basis for the transmission of the conformational changes from the allosteric site to the active site.

Binding of ADP induces no further conformational changes but enhances the CIT binding at the allosteric site. The previous biochemical studies showed that mammalian NAD-IDHs can be activated by ADP through decreasing the $S_{0.5,\text{ICT}}$ as well. Our biochemical data have also shown that the αγ heterodimer and the αβγγ heterotetramer can be activated by ADP, and additionally the activation effect of CIT and ADP together is more dramatic than CIT or ADP alone, indicating that the two activators work synergistically. To investigate the molecular mechanisms of the ADP activation and the synergistic effect of CIT and ADP, we sought but failed to obtain crystals of the αγ heterodimer bound with ADP alone at the allosteric site using either co-crystallization or soaking methods; however, in the presence of CIT and ADP, we obtained crystals and thus determined the structure of the αβγγ-MgICT+ADP heterodimer. In this structure, ADP binds next to CIT and Mg$^{2+}$ at the allosteric site and has both hydrophobic and hydrophilic interactions with several residues of the γ subunit (Fig. 3a). Specifically, the adenine moiety of ADP binds to a hydrophobic pocket composed of Ile26G, Pro252G, Ile278G, Gly253G and Ala284G, and additionally forms two hydrogen bonds with the main-chain amine and carbonyl of Asn285G at the deep end of the pocket. The α-phosphate of ADP forms hydrogen bonds with the side chain of Asn273G and the main-chain amine of Gly275G; and the β-phosphate forms hydrogen bonds with the side chains of Lys276G and Thr274G and the main-chain amine of Thr274G, and additionally makes a coordination bond with the Mg$^{2+}$.

Interestingly, comparison of the αβγγ-MgICT and αβγγ-MgICT+ADP structures shows that the key residues composing the allosteric site, the active site and the structure elements at the heterodimer interface assume almost identical conformations in both structures, indicating that the binding of ADP does not induce further conformational changes (Supplementary Figure S4). On the other hand, comparison of the αβγγ-MgICT and αβγγ-MgICT+ADP structures with the αβγγ structure shows that upon the CIT binding, several residues in the 312β–1αβ loop (residues 272G–276G) at the allosteric site undergo notable conformational changes to assume positions that are suitable not only for CIT or ADP alone, indicating that the two activators work synergistically. In particular, in the αβγγ structure, the side chain of Asn273G partially occupies the space for the side chains of Lys173G, Arg128G, Tyr135G, and Arg272G; the α-hydroxyl group makes hydrogen-bonding interactions with the side chains of Lys173G, Arg128G, Tyr135G, and Arg272G; the α-carboxyl group makes hydrogen-bonding interactions with the side chains of Lys173G and Asn175G; the β-carboxyl group makes hydrogen-bonding interactions with the side chains of Asn175G, Ser91G and Arg92G; and the γ-carboxyl group forms hydrogen-bonding interactions with the side chains of Asn78G and Asn93G and makes a coordination bond with the Mg$^{2+}$. Moreover, the residues composing the allosteric site, the active site and the structure elements at the heterodimer interface assume almost identical conformations as those in the αβγγ-MgICT+ADP structure (Supplementary Figure S4). The crystallization results and structural data together indicate that ADP cannot stably bind to the allosteric site by itself, and the binding of CIT (and Mg$^{2+}$) induces proper conformational changes of the allosteric site to facilitate or stabilize the binding of ADP and hence is likely to precede the ADP binding. On the other hand, the structural analysis shows that the binding of ADP establishes a more extensive network of hydrophilic and hydrophobic interactions among CIT, ADP and the surrounding residues mediated by the Mg$^{2+}$, which conversely enhances or stabilizes the CIT and Mg$^{2+}$ binding. This interplay of CIT and ADP provides the molecular basis for the synergistic activation effect of the two activators.

ICT can bind to the CIT-binding subsite and induces similar conformational changes. Our kinetic data showed that the αγ heterodimer exhibits a Hill coefficient of 2 for CIT in the absence of CIT but a Hill coefficient of 1 in the presence of CIT, indicating that the αγ heterodimer has two cooperative ICT-binding sites, one of which is blocked or occupied by CIT upon the CIT binding. In the absence of ADP, we failed to obtain crystals of the αγ heterodimer bound with ICT at the allosteric site using either co-crystallization or soaking methods. However, in the presence of both ICT and ADP, we obtained crystals and thus determined the structure of the αβγγ-MgICT+ADP heterodimer. In this structure, ICT binds to the CIT-binding subsite with a similar orientation as CIT and forms very similar hydrogen-bonding interactions with the surrounding residues (Fig. 3c). Specifically, the α-carboxyl group of ICT makes hydrogen-bonding interactions with the side chains of Lys173, Arg128, Tyr135, and Arg272; the α-hydroxyl group makes hydrogen-bonding interactions with the side chains of Lys173 and Asn175; the β-carboxyl group makes hydrogen-bonding interactions with the side chains of Asn175, Ser91 and Arg92; and the γ-carboxyl group forms hydrogen-bonding interactions with the side chains of Asn78 and Asn93 and makes a coordination bond with the Mg$^{2+}$. Moreover, the residues composing the allosteric site, the active site and the structure elements at the heterodimer interface assume almost identical conformations as those in the αβγγ-MgICT+ADP structure (Supplementary Figure S4). The crystallization results and structural data together indicate that like ADP, ICT cannot stably bind to the allosteric site alone, but can bind to the CIT-binding subsite in the presence of ADP which induces similar conformational changes as the binding of CIT and ADP. These results also imply that the binding of ICT (and ADP) should have an activation effect which is however weaker than the binding of CIT (and ADP). Intriguingly, our previous biochemical data...
Figure 3. Binding of ADP induces no further conformational changes. (a) Structure of the ADP-binding subsite in the \( \alpha_{Mg}^3 \cdot Mg \cdot CIT \cdot ADP \) structure. The protein is shown with electrostatic potential surface, the bound CIT and ADP are shown with ball-and-stick models, the \( Mg^{2+} \) with a green sphere, and the surrounding residues with side chains. The hydrophilic interactions of ADP with the surrounding residues and the \( Mg^{2+} \) are indicated with dashed lines. (b) Comparison of the ADP-binding subsite in the \( \alpha_{Mg}^3 \) (cyan), \( \alpha_{Mg}^3 \cdot CIT \) (orange) and \( \alpha_{Mg}^3 \cdot Mg \cdot CIT \cdot ADP \) (slate) structures. In the \( \alpha_{Mg}^3 \cdot CIT \) and \( \alpha_{Mg}^3 \cdot Mg \cdot CIT \cdot ADP \) structures, the side chain of Asn273G rotates about 100° away from the ADP-binding subsite compared to that in the \( \alpha_{Mg}^3 \) structure. (c) Comparison of the CIT-binding subsite in the \( \alpha_{Mg}^3 \cdot Mg \cdot ICT \cdot ADP \) (yellow) and \( \alpha_{Mg}^3 \cdot Mg \cdot CIT \cdot ADP \) (slate) structures. The bound ICT, CIT and ADP are shown with ball-and-stick models, the \( Mg^{2+} \) with a green sphere, and the surrounding residues with side chains. The hydrophilic interactions of ICT with the surrounding residues and the \( Mg^{2+} \) are indicated with dashed lines. ICT binds to the CIT-binding subsite and induces similar conformational changes.
Our structural data show that the S0.5,CIT of the αγ heterodimer is decreased by 1.7, 2.7, and 24.7 folds in the presence of CIT, ADP and both activators, respectively, suggesting that ADP has a slightly stronger activation effect than CIT, which seems to be in contradiction with the structural data showing that ADP cannot bind to the allosteric site alone but can bind to the allosteric site in the presence of CIT or ICT. This discrepancy can now be explained very well: because both ADP and ICT exist in the kinetic assay, the apparent activation effect of ADP is in fact the combined activation effect of ADP and ICT, which is slightly stronger than that of CIT alone but is much weaker than that of CIT and ADP. Moreover, our structural data suggest that the apparent S0.5,CIT of the αγ heterodimer in the absence of any activators contains the contribution of the weak activation of ICT.

Biochemical studies of the functional roles of the key residues. Our structural data show that the CIT binding induces conformational changes of a number of conserved residues at the allosteric site, the active site and the heterodimer interface, and the ADP binding does not induce further conformational changes but enhances the CIT binding via formation of a more extensive network of hydrogen-bonding interactions mediated by the metal ion. To validate the functional roles of these residues, we performed mutagenesis and kinetic studies to analyze their effects on the allosteric activation of the αγ heterodimer (Table 2).

Firstly, we analyzed the functional roles of the residues of the allosteric site involved in the CIT binding in the absence and presence of CIT (Table 2). In the absence of CIT, mutations of most of these residues slightly increase the S0.5,CIT by 1–2 folds and moderately decrease the catalytic efficiency (kcat/S0.5,CIT) by 3–10 folds compared to the wild-type enzyme. Mutation γ-R272A has a more severe effect on the S0.5,CIT (increased by 4.3 folds) and the catalytic efficiency (decreased by 10.0 folds). Mutations γ-N78A and γ-S91A are exceptions which have less critical effects on both the S0.5,CIT and the catalytic efficiency. These results support the notion that the binding of...
ICT alone has a weak activation effect, and mutations of most of the residues involved in the CIT binding at the allosteric site diminish the weak activation effect of ICT.

In the presence of CIT, the $S_{0.5,CIT}$ of the wild-type enzyme is decreased by 7.6 folds and the catalytic efficiency is increased by 12.9 folds, indicating a strong activation effect of CIT. In contrast, compared to these in the absence of CIT, the $S_{0.5,CIT}$ of most of the mutants is only decreased by 1–2 folds and the catalytic efficiency is only increased by 1–2 folds, indicating that mutations of these residues significantly impair the CIT activation effect (Table 2). Again, mutations γ−N78A and γ−S91A are exceptions which have less critical impacts on the CIT activation: mutation γ−N78A causes a 9.5-fold decrease in the $S_{0.5,CIT}$ and a 15.1-fold increase in the catalytic efficiency; and mutation γ−S91A causes a 4.0-fold decrease in the $S_{0.5,CIT}$ and a 8.9-fold increase in the catalytic efficiency. These results suggest that most of the residues involved in the CIT binding play an important role and Asn78 and Ser91 is a less essential role in the binding of CIT.

Secondly, we analyzed the functional roles of the residues of the allosteric site involved in the ADP binding in the absence and presence of ADP (Table 2). In the absence of ADP, mutations of these residues have less critical effects on the $S_{0.5,ICT}$ (increased by about 1.5 folds) and the catalytic efficiency (decreased by <2 folds), except for γ−N285A which severely impairs the enzymatic activity. In the presence of ADP, the wild-type enzyme exhibits a 2.7-fold lower $S_{0.5,ICT}$ and a 3.4-fold higher catalytic efficiency, indicating a moderate activation effect. Mutations γ−N273A and γ−T274A substantially compromise the activation effect of ADP, which cause only <1.2-fold decrease in the $S_{0.5,ICT}$ and <1.2-fold increase in the catalytic efficiency compared to these in the absence of ADP; however, mutation γ−K276A has a less critical effect on the ADP activation, which causes a 3.6-fold decrease in the $S_{0.5,ICT}$ and a 5.4-fold increase in the catalytic efficiency. As an exception, mutation γ−N285A completely abolishes the activity in the presence of ADP. These results suggest that Asn285 plays a critical role, Asn273 and Thr274 play an important role, and Lys276 play a less important role.

Thirdly, we analyzed the functional roles of the key residues at the heterodimer interface involved in the structural communication between the allosteric site and the active site in the absence and presence of CIT and ADP (Table 2). In the absence of the activators, mutations γ−E134A and α−E125A have significant effects on the $S_{0.5,ICT}$ (increased by <1.1 folds) and the catalytic efficiency (decreased by <2.1 folds); mutation α−D181A completely abolishes the activity; and the other mutations have moderate to severe effects on the $S_{0.5,ICT}$ (increased by 1.2–3.3 folds) and the catalytic efficiency (decreased by 5.0–47.0 folds). In the presence of CIT and ADP, the wild-type enzyme is significantly activated with the $S_{0.5,ICT}$ being decreased by 25.0 folds and the catalytic efficiency being increased by 44.5 folds. Again, mutations γ−E134A and α−E125A have no significant impact on the activation and these two mutants exhibit comparable $S_{0.5,ICT}$ and catalytic efficiency as the wild-type enzyme; mutation α−D181A completely abolishes the activity; and the other mutations severely impair or completely abolish the activation effect and these mutants exhibit a slightly decreased or increased $S_{0.5,ICT}$ (−2.4 to +1.2 folds) and catalytic efficiency (−1.2 to +4.5 folds) compared to these in the absence of the activators. These results suggest that Lys151, Asp190, Tyr237, Lys142, Asp181, and Tyr228 play an important role but Glu134 and Glu125 a less critical role in the transmission of the conformational changes from the allosteric site to the active site upon the binding of CIT and ADP.

Analyses of the kinetic data also show that the wild-type αγ heterodimer exhibits a Hill coefficient of 2 for ICT in the absence of CIT but a Hill coefficient of 1 in the presence of CIT or CIT and ADP (Table 2), indicating that the αγ heterodimer has two cooperative ICT-binding sites, one of which is occupied by CIT upon the CIT binding. Consistent with their effects on the $S_{0.5,ICT}$ and the catalytic efficiency, mutations of the key residues involved in the CIT binding except for the γ−N78A and γ−S91A mutations abolish the cooperativity in the absence of CIT because these mutations impair the binding of CIT at the allosteric site; and mutations of the key residues at the heterodimer interface except for the γ−E134A and α−E125A mutations also abolish the cooperativity in the absence of CIT and ADP because these mutations disrupt the structural communication between the allosteric site and the active site. Like the wild-type enzyme, all the mutants suppress the cooperativity in the presence of CIT or CIT and ADP. The exceptions are the γ−N78A, γ−S91A, γ−E134A and α−E125A mutants which exhibit a Hill coefficient of about 1.7 for ICT in the absence of CIT (and ADP), indicating the existence of two cooperative ICT-binding sites (Table 2). This is in agreement with the biochemical data showing that these mutations have no significant effects on the CIT binding and the CIT activation. On the other hand, the wild-type αγ heterodimer exhibits a Hill coefficient of 2 for ICT in the absence of ADP and a Hill coefficient of 1.6 in the presence of ADP (Table 2), indicating that the αγ heterodimer still has two ICT-binding sites with positive cooperativity in the presence of ADP. Furthermore, mutations of the residues involved in the ADP binding do not abolish the cooperativity (with the Hill coefficient of >1.5) in both the absence and presence of ADP. These results are consistent with the structural data showing that the allosteric site can bind ICT in the presence of ADP, and mutations of the residues involved in the ADP binding do not disrupt the binding of CIT (or ICT) at the allosteric site.

**Mutation γ−K151A disrupts the structural communication between the allosteric site and the active site.**

Our structural and biochemical data reveal that Lys151, Asp190, and Tyr237 of the γ subunit, and Lys142, Asp181, and Tyr228 of the α subunit at the heterodimer interface play critical roles in the transmission of the conformational changes from the allosteric site to the active site through the alteration of hydrogen-bonding interactions, and mutations of these residues abolish the activation effect of CIT and ADP (Fig. 2 and Table 2). To investigate the structural basis for the functional roles of these residues in the allosteric regulation, we took the γ−K151A mutation as a representative and determined the crystal structure of the αγK151A mutant bound with Mg$^{2+}$, CIT and ADP at the allosteric site (Table 1). In the αγK151A:Mg$^{2+}$:CIT:ADP structure, the structure of the allosteric site is very similar to that in the αMg$^{2+}$:Mg$^{2+}$:CIT:ADP structure with the CIT, ADP, and Mg$^{2+}$ binding to the allosteric site in similar manners and maintaining almost identical interactions (Fig. 4a,b and Supplementary Figure S4a,b). In addition, accompanying with the conformational changes of Arg97 and Tyr135, the N-terminal part of the β5−β6 loop and the N-terminal region of the αγ helix at the heterodimer
Figure 4. Mutation γ-K151A disrupts the structural communication between the allosteric site and the active site. (a) Comparison of the αMgγMg+ CIT+ADP (slate) and αγK151AMg+ CIT+ADP (green) structures. No major conformational changes are found at the allosteric site. However, significant conformational differences are observed in the C-terminal region of the β5–β6 loop and the β7 strand of both the α and γ subunits, the N-terminal region of the α7 helix of the α subunit, and the active site. (b) Comparison of the allosteric site in the αMgγMg+ CIT+ADP (slate) and αγK151AMg+ CIT+ADP (green) structures. The residues involved in the binding of CIT, Mg2+ and ADP adopt almost identical conformations in these two structures. The hydrogen-bonding interactions in the αMgγMg+ CIT+ADP structure are indicated with dashed lines. (c) Conformations of the structure elements at the heterodimer interface including the β5–β6 loop, the α7 helix and the β7 strand of both the α and γ subunits in the αγK151AMg+ CIT+ADP structure. The key residues involved in the structural communication between the allosteric site and the active site are shown with side chains, and the hydrogen-bonding interactions are indicated with dashed lines.
These results suggest that the apparent activation effect of ICT (and ADP) would have a weaker activation effect than the binding of CIT (and ADP). The conformational changes as the CIT binding, indicating that the allosteric site has a lower binding affinity for ICT than for CIT and hence enhances or stabilizes the CIT binding. Therefore, the binding of CIT and ADP together has a synergistic activation effect. Furthermore, our structural and biochemical data demonstrate that ICT cannot stably bind to the CIT-binding subsite alone, but can bind in the presence of ADP which induces similar conformational changes and catalytic efficiency. The binding of ADP in the presence of CIT does not induce further conformational changes at the allosteric site and the active site, but establishes a more extensive hydrogen-bonding network among CIT, ADP and the surrounding residues through the metal ion, which conversely enhances or stabilizes the CIT binding. Hence, the binding of CIT and ADP together has a synergistic activation effect, and the enzyme assumes the fully activated state which has a substantially decreased $S_{0.5}$ with a significantly increased catalytic efficiency.

Discussion

In this work, we determined a series of structures of the αγ heterodimer bound without or with the activator(s) (CIT, ICT and ADP) at the allosteric site. Analyses of those structures reveal the conformational changes at the allosteric site, the active site, and the heterodimer interface upon the binding of the activator(s) and identify the key residues involved in the transmission of the conformational changes from the allosteric site to the active site. The functional roles of these residues are validated by mutagenesis and kinetic data. The structural and biochemical data together demonstrate that CIT can bind independently to the allosteric site and the CIT binding induces significant conformational changes at the allosteric site, which are transmitted to the active site via the conformational changes of several structure elements at the heterodimer interface, including the $\beta_{5}$$\beta_{6}$ loop, the $\alpha 7$ helix, and the $\beta 7$ strand in both the $\alpha$ and $\gamma$ subunits, leading to the conversion of the active site from the inactive conformation to the active conformation favorable for the ICT binding. Hence, the enzyme assumes the partially activated state which has a moderately decreased $S_{0.5}$ with a moderately increased catalytic efficiency. The binding of ADP in the presence of CIT does not induce further conformational changes at the allosteric site and the active site, but establishes a more extensive hydrogen-bonding network among CIT, ADP and the surrounding residues through the metal ion, which conversely enhances or stabilizes the CIT binding. Hence, the binding of CIT and ADP together has a synergistic activation effect, and the enzyme assumes the fully activated state which has a substantially decreased $S_{0.5}$ with a significantly increased catalytic efficiency.
combined effect of ICT and ADP, explaining why the activation effect of ADP alone is slightly higher than that of CIT alone but is much weaker than that of CIT and ADP.

Based on our structural and biochemical data, we can propose the molecular mechanism for the allosteric regulation of the α7 heterodimer of human NAD-IDH (Fig. 5). The α7,γ5 structure represents the basal state of the enzyme. In this state, in both the α and γ subunits, the N-terminal region of the α7 helix adopts a loop conformation, and the β5–β6 loop interacts with the N-terminal region of the α7 helix and the β7 strand via several hydrogen bonds, but there is no direct interaction between the α7 helix and the β7 strand. Particularly, the side chain of Tyr126 at the active site assumes a conformation unsuitable for the ICT binding, and therefore the basal state of the enzyme has a high S0.5,ICT and hence a low catalytic efficiency.

The α6-Mg2+ + CIT structure represents the partially activated state of the enzyme. In this state, the binding of CIT induces substantially conformational changes of several key residues (particularly Tyr135) at the allosteric site, which further induce conformational change of the β5–β6 loop. Thus, several residues of the β5–β6 loop change their hydrogen-bond interactions with residues of the α7 helix and the β7 strand, which subsequently transduces the conformational changes of the α7 helix and the β7 strand to the heterodimer interface. Particularly, the hydrogen bond between the main-chain amine of Glu134 (the β5–β6 loop) and the main-chain carbonyl of Asn235 (the α7 helix) is disrupted and the N-terminal region of the α7 helix undergoes conformational change to transform from a loop conformation to an α-helical conformation. As a result, the side chain of Tyr237 at the (α7 helix) is in a proper position to form a tripartite hydrogen-bonding network with the side chains of Asp190 (the α5 helix) and Lys151 (the β7 strand). Concurrently, the hydrogen bond between the main-chain amine of Leu138 (the β5–β6 loop) and the main-chain carbonyl of Leu150 (the β7 strand) is also disrupted and a new hydrogen bond is formed between the main-chain carbonyl of Gly133 (the β5–β6 loop) and the main-chain amine of Ile129 (the β7 strand). These two aspects together stabilize the interactions of the β5–β6 loop, the α7 helix and the β7 strand and thus induce and/or stabilize the bending of the β7 strand around residues Ser149–Lys151.

The conformational changes of the allosteric site and the structure elements at the heterodimer interface in the γ subunit are then transmitted to the α subunit and the active site in a pseudo symmetric manner. In the αγ heterodimer, the β6 and β7 strands of the clasp domain of the α and γ subunits form a four-stranded anti-parallel β-sheet at the heterodimer interface, and the extensive hydrogen-bonding interactions between main chains of the residues of the β6 and β7 strands form the core of the β-sheet. The bending of the β7 strand around residues Ser149–Lys151 upon the CIT binding induces the bending of the β7 strand around residues Ser140–Lys142 towards the β5–β6 loop and the α7 helix. This conformational change breaks up the hydrogen-bonding interaction between the main-chain carbonyl of Ile141 (the β7 strand) and the main-chain amine of Ile129 (the β5–β6 loop) and forms a new hydrogen-bonding interaction between the main-chain amine of Leu143 (the β7 strand) and the main-chain carbonyl of Gly124 (the β5–β6 loop), which induce the conformational change of the β5–β6 loop. Subsequently, the hydrogen-bonding interaction between the main-chain amine of Glu125 (the β5–β6 loop) and the main-chain carbonyl of Asn226 (the α7 helix) is disrupted and the N-terminal region of the α7 helix undergoes conformational change to transform from the loop conformation to the helical conformation. Consequently, the side chain of Lys142 (the β7 strand) forms a tripartite hydrogen-bonding network with the side chains of Try228 (the α7 helix) and Asp181 (the α5 helix). The alterations of the hydrogen-bonding interactions among the β5–β6 loop, the β7A strand) and the main-chain amine of Glu125 (the β5–β6 loop) and the main-chain carbonyl of Asn226 (the α7 helix) is disrupted and the N-terminal region of the α7 helix further stabilize the conformational changes of the β5–β6 loop. As a result, the side chain of Tyr126 (the β5–β6 loop) undergoes conformational change and assumes a conformation suitable for the ICT binding at the active site, and hence the partially activated state of the enzyme has a moderately decreased S0.5,ICT and a moderately increased catalytic efficiency.

The α6-Mg2+ + CIT + ADP structure represents the fully activated state of the enzyme. In this state, the ADP binding does not induce further conformational changes at the allosteric site and the active site, but establishes a more extensive hydrogen-bonding network among CIT, ADP and the surrounding residues through the metal ion and stabilizes the CIT and ADP binding with each other, which enhances the structural communication between the allosteric and active sites and further stabilizes the ICT binding at the active site. Therefore, the binding of CIT and ADP together has a synergistic activation effect, and the fully activated state of the enzyme has a substantially decreased S0.5,ICT and a significant increased catalytic efficiency.

Yeast NAD-IDH consists of the IDH1/IDH2 heterodimer as the basic structural and functional unit, which is allosterically regulated by CIT and AMP21,22. The crystal structures of yeast NAD-IDH show that the binding of CIT at the allosteric site could induce conformational changes of the active site and thus enhances the binding affinity for ICT21. Yeast IDH1/IDH2 heterodimer has a similar structural topology as human αγ heterodimer. In addition, sequence alignment of yeast IDH1 and IDH2 subunits and human α and γ subunits shows that residues at the allosteric site, the active site, and the heterodimer interface are largely strictly conserved (Supplementary Figure S5). Thus, we performed a detailed structural comparison of yeast IDH1/IDH2 and human αγ heterodimers, which shows that the two heterodimers assume very similar structures at the allosteric site, the active site and the heterodimer interface in both the apo and CIT-bound structures; however, there are some conformational differences (Supplementary Figure S6). Specifically, in the apo IDH1/IDH2 heterodimer, residues 78–92 at the allosteric site assume a helical conformation which spatially occupies the CIT-binding site, and the N-terminal region of the α7 helix in both IDH1 and IDH2 subunits at the heterodimer interface adopts a helical conformation; the CIT binding induces structural change of residues 78–92 from the helical conformation to a loop conformation but does not induce structural change of the N-terminal region of the α7 helix. In the apo αγ heterodimer, residues 77–91 at the allosteric site assume a loop conformation which does not block the CIT-binding site, and the N-terminal region of the α7 helix in both α and γ subunits at the heterodimer interface assumes a loop conformation; the CIT binding does not induce structural change of residues 77–91 but induces structural change of the N-terminal region of the α7 helix from the loop conformation to a helical conformation.
Moreover, structural comparison of the apo and CIT-bound IDH1/IDH2 heterodimer shows that upon the CIT binding, the allosteric site, the active site and the heterodimer interface undergo conformational changes in similar manners as those in human αγ heterodimer (Supplementary Figure S7). In the apo IDH1/IDH2 structure, the CIT-binding site of the IDH1 subunit is spatially occupied by residues 78–92 with a helical conformation (Supplementary Figure S7a,b). Meanwhile, the side chain of Arg98IDH1 (corresponding to Arg97G) forms a cation–π interaction with the side chain of Phe136IDH1 (corresponding to Tyr135G), making the side chains of Arg98IDH1 and Phe136IDH1 point away from the CIT-binding site. At the heterodimer interface, the 35–36 loop in both IDH1 and IDH2 subunits adopts a conformation similar to that in the αMγ structure and interacts with the N-terminal region of the α7 helix via several hydrogen bonds; the α7 strand does not bend towards the α7 helix and there is no hydrogen-bonding interaction between the two structure elements (Supplementary Figures S6 and S7a,c,d). At the active site of the IDH2 subunit, the side chain of Tyr142IDH2 (corresponding to Tyr126A) assumes a similar conformation as that of Tyr126A in the αMγ structure.

In the CIT-bound IDH1/IDH2 structure, residues 78–92 at the allosteric site adopt a loop conformation; the cation–π interaction between the side chains of Arg98IDH1 and Phe136IDH1 is disrupted, and consequently the side chain of Arg98IDH1 assumes a different conformation to form a hydrogen bond with the γ-carboxyl of CIT, and the side chain of Phe136IDH1 is rotated towards and makes van der Waals contacts with the γ-carboxyl of CIT (Supplementary Figure S7a,b). In addition, the β5IDH1–β6IDH1 loop adopts a similar conformation as that in the αMγ–CIT structure and several residues of this loop change their hydrogen-bonding interactions with residues of the (β7IDH1 strand (Supplementary Figures S6 and S7c,d). These changes lead to the formation of a tripartite hydrogen-bonding network among the side chains of Lys152IDH1 (corresponding to Lys151G), Tyr237IDH1 (corresponding to Tyr237A), and Asp191IDH1 (corresponding to Asp190G), which further facilitate and stabilize the conformational changes at the allosteric site and the heterodimer interface of the IDH1 subunit are transmitted to the IDH2 subunit and the active site in a pseudo symmetric manner. Through the extensive hydrogen-bonding interactions between the (β7IDH1 strand and the (β5IDH1 strand in the four-stranded β-sheet at the heterodimer interface, the (β7IDH1 strand is induced to bend towards the α7IDH2 helix and concurrently the β5IDH1–β6IDH1 loop adopts a conformation similar to that in the αMγ–CIT structure (Supplementary Figure S7a). These conformational changes lead to the disruption of several hydrogen bonds and the establishment of a more extensive hydrogen-bonding interaction network among the (β7IDH1 strand, the β5IDH1–β6IDH1 loop and the α7IDH2 helix (Supplementary Figure S7c,d). In particular, the side chain of Lys152IDH1 (corresponding to Lys152A) forms a tripartite hydrogen-bonding network with the side chains of Tyr246IDH1 (corresponding to Tyr228A) and Asp197IDH1 (corresponding to Asp181A). As a result, the side chain of Tyr142IDH2 (corresponding to Tyr128A) is rotated towards the active site and assumes a conformation favorable for ICT binding. Furthermore, similar to the αγ heterodimer, structural comparison between the CIT-bound and CIT+AMP-bound IDH1/IDH2 shows that the binding of CIT creates the AMP-binding site and the binding of AMP does not induce further conformational changes at the allosteric site and the active site.

These results together indicate that yeast IDH1/IDH2 heterodimer and human αγ heterodimer use a similar molecular mechanism for structural communication between the allosteric site and the active site, and thus share a common allosteric regulation mechanism. Furthermore, sequence alignment of human and yeast NAD-IDHs with other eukaryotic NAD-IDHs from Arabidopsis thaliana, Danio rerio, and Xenopus laevis shows that the residues composing the allosteric site, the active site and the heterodimer interface, and especially those involved in the conformational changes upon the binding of CIT (and ADP/AMP) are also largely strictly conserved (Supplementary Figure S5), suggesting that the other eukaryotic NAD-IDHs are likely to utilize a similar allosteric regulation mechanism as human αγ heterodimer.

**Methods**

**Cloning, expression, and purification.** The αγ heterodimer of human NAD-IDH was prepared as described previously40. Briefly, the DNA fragments encoding the α and γ subunits of human NAD-IDH were cloned into the co-expression vector pQlinkN with C-terminal of the γ subunit attached with a TEV protease cleavage site and a His6 tag following the pQlink cloning procedure41. The pQlinkN-α-γ-tev-His6 plasmid was transformed into E. coli BL21(DE3) Codon-Plus strain (Novagen). When the culture of the transformed cells reached an OD600 of 0.5, 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 the lysis buffer [50 mM HEPES-Na, pH 7.4, 200 mM NaCl, 0.2 mM MnCl2, 10% (v/v) glycerol, and 7.2 mM 3-ME] supplemented with 1 mM PMSF. The target protein was 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 and elution buffers, respectively. The elution fraction was dialyzed overnight against the lysis buffer supplemented with proper amount of TEV protease to lower the concentration of imidazole to <10 mM and to elute the His6-tag of the target protein. The protein mixture was reloaded on a Ni-NTA column and washed with the lysis buffer supplemented with 10 mM imidazole. The flow-through fraction contains the target protein, which was further purified by gel filtration using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with the storage buffer (10 mM HEPES, pH 7.4, 200 mM NaCl, and 5 mM 3-ME). The purity of the protein was assessed by 12% SDS-PAGE. The purified protein was concentrated to 10 mg/ml and stored for further structural and biochemical studies.

Mutants of the αγ heterodimer containing point mutations in the α and γ subunits were constructed using the QuickChange® Site-Directed Mutagenesis kit (Strategene). Expression and purification of the mutants were carried out the same as for the wild-type protein.
Enzymatic activity assay. The enzymatic activity of the αγ heterodimer was determined by monitoring the 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, pH 7.4, 2 ng/ml enzyme, 80 mM Mg²⁺-ICT, 2 mM ADP, and 3.2 mM NAD. The reaction was initiated by addition of NAD. The specific activity is defined as the amount of NADH produced per minute per milligram of enzyme (μmol/min/mg) at the standard conditions. Kinetic data in the absence of activators were measured at the standard conditions with varied concentrations of αγ-Mg²⁺-ICT (0–80 mM) to obtain the Vmax and S0.5 values for ICT. Kinetic data in the presence of activator(s) were determined at the above conditions in the presence of given concentrations of CIT or/and ADP. Kinetic parameters were obtained by fitting the data into the Non-Michaelis-Menten equation \( V = \frac{V_{\text{max}}[S]}{S_0 + [S]} \) using the Graphpad Prism program (Graphpad Software), where \( S_0 \) is the Hill coefficient, \( S_0 \) is the apparent Km value for ICT (the ICT concentration at which the reaction rate is 0.5 * \( V_{\text{max}} \)), and \([S]\) is the concentration of ICT. All the experiments were performed in three independent measurements and the values were the averages of the three measurements with the standard errors.

Crystallization and diffraction data collection. Crystallization was performed using the hanging drop vapor diffusion method at 20 °C by mixing equal volume (1 μl) of protein solution (10 mg/ml) and reservoir solution. Crystals of the αγ heterodimer without any activators bound to the γ subunit (αMG-γ) were grown from drops with the reservoir solution containing 0.1 M HEPES-Na, pH 7.5, 50 mM MgCl₂, and 30% (v/v) PEGMME 550. Crystals of the αγ heterodimer with Mg²⁺ and CIT bound to the γ subunit (αMG-γ-MG-ICT) were grown from drops consisting of the protein solution supplemented with 0.2 mM Mg²⁺ and the reservoir solution containing 0.2 M sodium citrate, pH 8.0, and 20% (v/v) PEG3350. Crystals of the αγ heterodimer with Mg²⁺, CIT and ADP bound to the γ subunit (αMG-γ-MG-ICT-ADP) were grown from drops consisting of the protein solution supplemented with 0.2 mM Mg²⁺ and 2 mM ADP and the above reservoir solution. Crystals of the αγ heterodimer with Mg²⁺, ICT and ADP bound to the γ subunit (αMG-γ-MG-ICT+ADP) were grown from drops consisting of the protein solution supplemented with 0.2 mM Mg²⁺ and 2.2 mM ADP and the reservoir solution containing 0.1 M HEPES-Na, pH 7.5, and 12% (v/v) PEG 3350. Crystals of the mutant αγ(K151A) heterodimer with Mg²⁺, ICT and ADP bound to the γ subunit (αMG-γ-MG-ICT+ADP) were grown at the same conditions as for the crystals of the αMG-γ-MG-ICT+ADP heterodimer. Prior to diffraction data collection, the crystals were cryoprotected using the reservoir solution supplemented with 25% ethylene glycol and then flash-cooled into liquid N₂. Diffraction data were collected at 100 K at BL19U1 of the National Facility for Protein Science in Shanghai and processed with HKL2000. Statistics of the diffraction data are summarized in Table 1.

Structure determination and refinement. All structures were determined with the molecular replacement (MR) method as implemented in program Phaser. The αMG-γ structure was solved using the structure of HcIDH bound with NADP (PDB code 1T09) as the search model. The αMG-γ-MG-ICT structure was solved using the αMG-γ-MG-ICT structure as the search model, which was successively used as the search model to solve the αMG-γ-MG-ICT+ADP, αMG-γ-MG-ICT+ADP and αMG-γ-MG-ICT+ADP structures. Initial structure refinement was carried out with program Phenix and final structure refinement was performed with program REFMAC5. Model building was performed with program Coot. Stereochemistry and quality of the structure models were analyzed using programs in the CCP4 suite and the PISA server. All structure figures were prepared using PyMol. The structure-based sequence alignment figures were prepared using ESPript. Statistics of the structure refinement and the final structure models are summarized in Table 1.

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Author Contributions

T.M. carried out the structural and biochemical studies, and drafted the manuscript. Y.P. and W.H. participated in the initial structural studies. J.D. conceived the study, participated in the experimental design, data analyses and discussion, and wrote the manuscript.
Additional Information
Accession codes: The $\alpha$Mg$\gamma$, $\alpha$Mg$\gamma$Mg-CIT, $\alpha$Mg$\gamma$Mg+ADP, $\alpha$Mg$\gamma$ICT+ADP, and $\alpha\gamma K151A$Mg+CIT+ADP structures have been deposited in the Protein Data Bank with accession codes 5GRH, 5GRI, 5GRE, 5GRL, and 5GRF, respectively.

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